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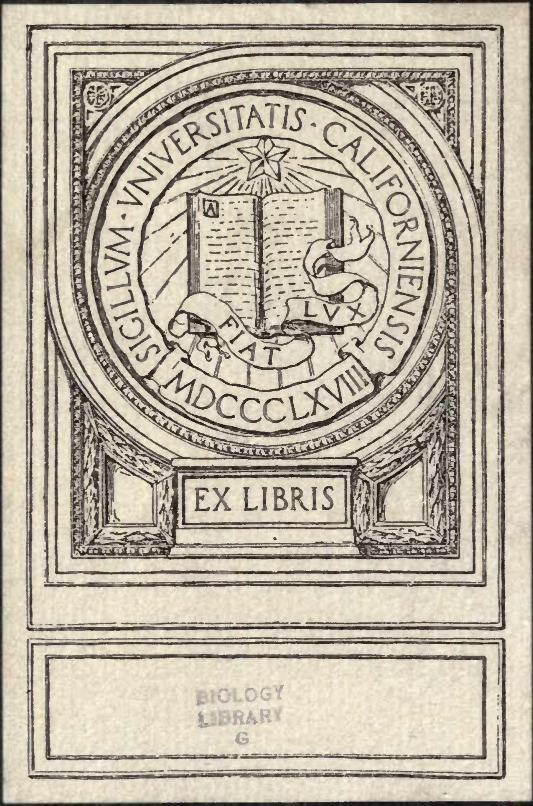
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A LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

WITH SUPPLEMENT

BY

OTTO FOLIN

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NEW YORK AND LONDON
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PREFACE TO SECOND EDITION

For this edition the manual has been very largely rewritten. Much painstaking research is represented in the revised analytical methods here given for the first time in book form, and if the directions are followed these methods give reliable results.

Much valuable assistance has been received from Dr. C. H. Fiske in connection with this revision.

OTTO FOLIN

BOSTON

PREFACE

This manual of biological chemistry for medical students in Harvard Medical School has been revised annually for the past seven years, and it is believed now to meet our needs sufficiently well to warrant publication.

For many years I have been interested in the development of analytical methods applicable to metabolism investigations. The most serviceable of my older methods and some of the newer methods have been taught to our medical students; these are described in the main body of the manual. Others not heretofore included have been incorporated in the supplement, so that nearly all the newer methods devised in the department are now described in this manual.

In connection with the revisions referred to above I am indebted for valuable help to W. R. Bloor, W. Denis, C. J. Farmer, L. J. Morris, F. B. Kingsbury, F. S. Hammett, R. D. Bell, and C. H. Fiske, as well as to my older friend, P. A. Shaffer.

OTTO FOLIN

BOSTON

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LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

PART I

ACIDIMETRY, ALKALIMETRY, NITROGEN DETERMINATION

Equivalent and Normal Solutions.—Since the molecular weight of sodium hydroxid ($NaOH$) is 40 and that of hydrochloric acid (HCl) is 36.46, it follows that 40 g. of the former contain the same number of molecules as 36.46 g. of the latter. If 40 g. of sodium hydroxid and 36.46 g. of hydrochloric acid are each dissolved in pure water sufficient to make one liter of solution, each liter will contain the same number of dissolved molecules.

It will take a little less than one liter of water to make a liter of solution because the dissolved substance takes up some space. A normal sodium hydroxid solution contains four per cent. of sodium hydroxid. By per cent. in the case of solutions is usually meant the amount of substance present in 100 c.c. of solution.

Mixing equal volumes of two such solutions is, therefore, the same as bringing together practically the same number of the two kinds of molecules, and the result is the instantaneous and essentially complete transformation into sodium chlorid (and water).

$$X\,NaOH + X\,HCl = X\,NaCl + X\,H_2O$$

If either or both of the solutions should first be diluted with a considerable bulk of pure water, the result on mixing the two would be the same, for the extra amount of water present takes no part in the reaction (except to the extent of absorbing a part of the heat set free).

The two solutions are equivalent. They also happen to be normal solutions. The hydrochloric acid is normal because it con-

tains 1 g. of active or replaceable hydrogen per liter of solution, and not because it contains the same number of grams of HCl per liter as there are units in the molecular weight. The sodium hydroxid solution is normal because it is equivalent to a solution containing one gram of replaceable hydrogen per liter.

The molecular weight of sulphuric acid is 98. A sulphuric acid solution containing exactly 98 g. per liter contains, therefore, the same number of molecules per unit volume as the sodium hydroxid solution containing 40 g. per liter. But one molecule of sulphuric acid requires two molecules of sodium hydroxid for the formation of the neutral salt, sodium sulphate, because the sulphuric acid molecule has two replaceable hydrogen atoms. The solutions are not equivalent, for the sulphuric acid contains 2 g. active hydrogen per liter. It is exactly twice as strong as the sodic hydrate solution; it is a 2 normal solution.

On the basis of the above description of what constitutes a normal solution, calculate the number of grams per liter in tenth normal sulphuric acid (.1N H_2SO_4), fifth normal hydrochloric acid (.2N HCl), half normal oxalic acid (.5N $C_2H_2O_4$, $2H_2O$), fourth normal acetic acid (.25N CH_3COOH), half normal sodic hydrate (.5N NaOH), twentieth normal barium hydrate (.05N $Ba(OH)_2$), fifth normal ammonium hydrate (.2N NH_4OH).

Atomic weights of some of the more important elements: Arsenic (As) 74.96, Barium (Ba) 137.37, Bromin (Br) 79.92, Calcium (Ca) 40.09, Carbon (C) 12, Chlorin (Cl) 35.46, Copper (Cu) 63.57, Hydrogen (H) 1.008, Iodin (I) 126.92, Iron (Fe) 55.85, Lead (Pb) 207.1, Magnesium (Mg) 24.32, Manganese (Mn) 54.93, Mercury (Hg) 200.6, Nitrogen (N) 14.01, Oxygen (O) 16, Phosphorus (P) 31.04, Potassium (K) 39.1, Sulphur (S) 32.07, Tin (Sn) 119, Tungsten (Wo) 184, Uranium (U) 238.5, Zinc (Zn) 65.37.

The same description of normal solutions applies to other substances than acids and alkalis, as, for example, reducing and oxidizing substances such as potassium permanganate, potassium bichromate, iodin, cupric hydrate, stannous chlorid. A normal solution is here one capable of liberating 1 g. of reducing hydrogen (or of giving off exactly sufficient oxygen to oxidize one gram of hydrogen) per liter. Potassium permanganate, for example, in the presence of sulphuric acid and some easily oxidizable substance is decomposed as follows:

$$2KMnO_4 + 3H_2SO_4 = K_2SO_4 + 2MnSO_4 + 3H_2O + 5O.$$

As the two permanganate molecules liberate oxygen enough for ten hydrogen atoms it takes only one fiftieth of the molecular weight expressed in grams (3.161 g.) to make one liter of tenth normal solution.

The calculation of what constitutes normal or equivalent solutions of any reagent is not very difficult provided the equation representing the chemical reaction involved is thoroughly clear.

To determine whether a given unknown solution is acid or alkaline it is usually sufficient to dip a piece of delicate violet colored litmus paper into it. (If the solution is acid the test paper turns red; if alkaline it turns blue.) Litmus, the substance with which the paper has been impregnated, is a complex organic product, and is one of the most familiar representatives of a most useful class of organic compounds which are so sensitive to acids or alkalis, or both, that they clearly and unmistakably indicate the presence of free acid or alkali even when the amounts present are so small as to be practically unweighable. By means of such *indicators* and accurate measuring instruments (measuring flasks, burets, and pipets), it becomes a simple matter to determine (by titration) the relative concentration or equivalence of acid and alkaline solutions. By their help it is possible to prepare with very little labor normal or tenth normal solutions, even of acids or alkalis which cannot be weighed on the balance, as for example, hydrochloric acid and ammonia, both of which are gases. Before this can be done we must, however, possess one normal or standard solution prepared from some substance which can be weighed.

Volumetric analysis consists of measuring the value of an unknown solution in terms of another the value of which is known (titration). The known solution is prepared directly or indirectly by the help of the analytical balance, and the first step in any kind of volumetric analysis is the preparation of the standard solution by means of which the values of others are to be determined.

Every student who has had no experience in the use of the analytical balance must consult the instructor before proceeding. He should also ask for instruction as to the proper use of measuring flasks, pipets, and burets before using them. He must particularly learn when the presence of unmeasured quantities of water does not interfere with the accuracy of the work and when a single drop of

unmeasured water introduces a perceptible error. (*See* Sutton's Volumetric Analysis, Part 1—"Instruments and Apparatus.")

All the common mineral acids and strong alkalis contain so much water that it is in practice not feasible to weigh out with sufficient accuracy the theoretical quantity required for a standard solution of acid or alkali. The carbonates of sodium or calcium (or the carbonates of sodium or potassium, obtained by ignition of the corresponding oxalates) give exceedingly accurate results. Oxalic acid is very serviceable as starting material for the preparation of standardized solutions of acids and alkalis if it is pure and has lost none of its water of crystallization.

1. Calibrations.—The volumetric ware now available is not always accurate enough for the work of this course. Some approximate calibrations are therefore necessary.

At least 1 pipet should be calibrated by weight. Clean a 20 c.c. pipet with "cleaning fluid" and rinse. Weigh a clean and empty but not necessarily dry 100 c.c. volumetric flask within an accuracy of 2 mg. Fill the pipet, adjust the lower part of the meniscus exactly at the mark, and transfer the contents to the weighed flask allowing to drain for 15 seconds against the inside of the flask. Weigh again to within an accuracy of 2 mg. 1 c.c. of water may be assumed to weigh 997 mg. The slight fluctuations due to variations in room temperature may be neglected. Calculate the correct volume of the pipet. Clean a glass stoppered buret with cleaning fluid, rinse, fill to the mark with distilled water, empty down to the 25 c.c. mark and let drain for 2 minutes. Then adjust the meniscus exactly to the 25 c.c. mark and be sure that the sides of the buret above the water are entirely free from drops of adhering water. Now run in 20 c.c. from the calibrated pipet. Compare the reading obtained with the correct value. If the divergence seems large consult the instructor. Next calibrate the 5 c.c. pipet by transferring its contents 5 times to the buret, beginning with the meniscus in the latter at the 25 c.c. mark. Record each reading but note particularly where the fourth comes. If the 5 c.c. pipet is inaccurate make a new temporary mark on the stern and repeat. Incidentally record the value of each 5 c.c. portion of the buret. The 10 c.c. and the 25 c.c. pipets can also be calibrated by the help of the buret.

For calibrating the larger pipets, calibrate first 1 dry 100 c.c. volumetric flask with the most accurate 20 or 25 c.c. pipet, making if necessary a new temporary mark on the stem of the flask. When this flask is again dry use it for checking up the values of a 50 or a 100 c.c. pipet. The volumetric flasks larger than 100 c.c. need not be calibrated. If a flask is rinsed with a little alcohol and left to drain over night it will usually be found to be perfectly dry the following day.

2. Preparation of .5N Oxalic Acid (500 c.c.).—The usefulness of oxalic acid as a starting point for the preparation of standard acids and alkalis is due entirely to the fact that it can be obtained chemically pure and in condition suitable for direct weighing. Oxalic acid is, however, not a strong enough acid to titrate well with all the common indicators, and it is therefore not serviceable for acidimetric titrations in general. But by means of oxalic acid and with phenolphthalein as indicator, standard solutions of a strong alkali, like caustic soda, can be obtained, and by means of the latter standard solutions of the stronger mineral acids can then be prepared.

The reason why the strong acids and alkalis give more accurate and reliable results is the fact that the salts which they form when neutralized are not appreciably hydrolyzed by water into acid and base, as are the corresponding salts of the weaker acids and bases. The zone of neutrality to different indicators is therefore more sharply defined, and corresponds more nearly to the point represented by the presence of exactly equivalent amounts of acid and alkali.

Weigh accurately (to the fourth decimal) a small, clean, and dry beaker or large crucible. Then add to the weights on the balance pan 15.7560 g., and add oxalic acid to the vessel on the other side until exact equilibrium is reached. Dissolve in distilled water this oxalic acid without the loss of a single crystal. The acid dissolves rather slowly. The solution is, therefore, best made in a beaker by the aid of gentle heating with about 250 c.c. water. Transfer every drop of the solution to a measuring flask (500 c.c.), carefully rinsing the last traces from the beaker into the flask by means of successive small amounts of cold distilled water. Cool the flask in running tap water until the contents of the flask have reached the room temperature. (If a thermometer is used it must be rinsed carefully before it is removed from the flask.) Fill up with water until the lower side of the "meniscus" is exactly even with the 500 c.c. mark. Stop-

per the flask, and invert several times (30-40) so that the solution is thoroughly mixed. Transfer to a clean, *dry* bottle; label and preserve.

Using a strong base like sodium hydroxid and a sensitive indicator like phenolphthalein for the titration, it is possible to obtain quite reliable and accurate results with oxalic acid. The volumetric determinations involved in metabolism studies and urine analysis are, however, extensively based on titrating ammonia, which is a very weak base. Phenolphthalein, because of its high degree of sensitiveness to weak acids and its lack of sensitiveness to weak bases, is useless in titrations of ammonia. The oxalic acid and the phenolphthalein are therefore used only for the purpose of securing a standard alkali solution.

3. Preparation of Standardized Sodium Hydroxid.—The sodium hydroxid used for titrations must be as free as possible from carbonates, because otherwise the solutions will not have the same titrating value with all the common indicators. Sodium hydroxid absorbs rapidly carbonic dioxid from the atmosphere and should therefore not be exposed to the air more than is unavoidable. As the carbonates are insoluble in very strong sodium hydroxid solutions, clear saturated solution should be used as starting point for the preparation of standard solutions.

Transfer about 60 c.c. of clear saturated sodium hydroxid solution to a large bottle and add 1,200 to 1,500 c.c. of water. To determine the exact value of this solution it is only necessary to find out how much of it is required for the neutralization of a known volume of the half normal oxalic acid solution.

Rinse the 20 c.c. pipet with the oxalic acid solution and then measure 20 c.c. into a beaker or flask. Add two drops of indicator (1 per cent. alcoholic solution of phenolphthalein).

Rinse a buret with the alkali, fill it and cover with a test tube. After carefully adjusting the meniscus of the solution to the zero point, run it into the oxalic acid solution more and more cautiously toward the end until finally one single drop produces a definite and stable end point. Note the volume of alkali required (within 0.05 c.c.). Repeat the titration until two successive ones give exactly the same value.

From the titration figure obtained calculate the normality of the solution and how much of it must be taken for the preparation of 1 liter of tenth normal alkali.

As a check on the work determine the normality of an un-

known hydrochloric acid solution (furnished), using as indicator (a) phenolphthalein, (b) alizarin red (2 drops of 1 per cent. aqueous solution). A dated and signed report on the unknown should be handed in before making the tenth normal alkali. Label and preserve the standardized alkali solution.

4. Standardized Hydrochloric Acid.—Concentrated hydrochloric acid is approximately a 10 N solution of HCl. With a cylinder transfer 60 c.c. of strong hydrochloric acid to a large bottle; add 1,500 c.c. of water and shake very thoroughly. It is preferable but not absolutely necessary to let the shaken solution stand over night before titrating.

Titrate this acid in the same way as the oxalic acid solution, but using only alizarin red as indicator. Calculate the normality, and how much of it must be taken for the preparation of 1 liter of tenth normal acid. Label and preserve.

5. Tenth Normal Acid and Alkali.—From the standardized solutions of acid and alkali prepare 1 liter of tenth normal hydrochloric acid and 1 liter of tenth normal alkali. Titrate the acid so prepared (20 c.c.) with the tenth normal alkali. The two should be equivalent. Determine the normality of an unknown acid with the tenth normal alkali. Hand in a dated and signed report giving the value obtained for the unknown and giving also the titration figures for the tenth normal acid.

Label and preserve the tenth normal solutions. The two alkali solutions do not always keep their value unchanged because more or less alkali is given off by the glass containers. The hydrochloric acids solutions keep indefinitely. If discrepancies are found later between the acid and the alkali, the acid should be taken as correct.

6. Strong and Weak Acids; the Use of Different Indicators. (A) Titrate 25 c.c. tenth normal hydrochloric acid with the tenth normal alkali, using as indicator (a) phenolphthalein (b) methyl orange (c) alizarin red. Repeat the above mentioned three titrations in the presence of 10 c.c. ammonium chlorid solution (2 per cent.). Repeat the titration with each indicator using in place of the hydrochloric acid (a) 25 c.c. .1N phosphoric acid (b) 25 c.c. .1N lactic acid.

Record the titrations in tabular form:

Phenolphthalein:	Methyl orange:	Alizarin red:
c.c.—End point.*	c.c.—End point.*	c.c.—End point.*

* Sharp, fair or indeterminate.

HCl:
HCl NH_4Cl:
Phosphoric acid:
Oxalic acid:

(B) Dilute 10 c.c. tenth normal hydrochloric acid to 100 c.c., making an approximately 0.01 N solution. (Measuring cylinders are accurate enough for the dilutions referred to here.)

From this 0.01 N solution prepare four 100 c.c. portions of more dilute acids, viz.: 0.001 N; 0.0004 N; 0.0001 N; 0.00001 N. Arrange in a row four test tubes, as nearly as possible of the same size, and transfer to each one 5 c.c. of one of the four dilute acid solutions. To the contents of each tube add one drop (no more) of a 0.15 per cent. alcoholic solution of tetrabromophenolsulfonephthalein ("bromphenol blue"), and compare the colors. The approximate hydrogen concentrations of these solutions are as follows:

	C_H	p^H[*]
0.001 N............	10^{-3}	3.0
0.0004 N...........	4×10^{-4}	3.4
0.0001 N...........	10^{-4}	4.0
0.00001 N..........	10^{-5}	5.0

Add the same amount of indicator to (a) 5 c.c. 0.001 N lactic acid, (b) 5 c.c. 0.001 N acetic acid, (c) 5 c.c. 0.001 M monopotassium phosphate. Determine the approximate p^H of each of these three solutions by comparing their colors with the dilute hydrochloric acid solutions. Although the total acid concentration is the same in the 0.001 N solutions of hydrochloric, lactic, and acetic acids, and monopotassium phosphate (an acid salt), the hydrogen ion concentration (and therefore the degree of dissociation) is obviously different in each case. In any such series of acid solutions of the same total concentration (0.001 N in this instance), the hydrogen ion concentration is less (and the p^H greater) the weaker the acid. The strength of an acid is measured by its dissociation constant (k), a figure approximately equal to the hydrogen ion concentration of a solution of the acid that has been just half neutralized. The dissociation constants of the three weak acids used in this experiment are given below,

---

[*] The hydrogen exponent (p^H) is the logarithm of the hydrogen ion concentration (C_H) with the minus sign omitted.

along with those of other acids and bases of biological importance.

Hippuric acid	2.2x10-4	Uric acid	1.5x10-6
Acetoacetic acid	1.5x10-4	Carbonic acid	3.0x10-7
Lactic acid	1.4x10-4	Primary phosphate	2.0x10-7
Acid oxalate	3.0x10-5	Boric acid	6.6x10-10
β-Hydroxybutyric acid	2.0x10-5		
Acetic acid	1.8x10-5	Ammonia	1.8x10-5

7. Acidity of Gastric Contents.—The acidity of the normal stomach contents is due almost wholly to hydrochloric acid. In pure gastric juice, the concentration of hydrochloric acid is about 0.15 N, but the acidity of the material usually found in the stomach is less, as a result of dilution and partial neutralization. When, under abnormal conditions, the concentration of hydrochloric acid becomes very low, certain microörganisms are able to grow in the stomach contents, producing lactic acid. It is nevertheless an easy matter to distinguish between a relatively low concentration of hydrochloric acid and a relatively high concentration of lactic acid, since the latter is a much weaker acid.

To 5 c.c. 0.01 N hydrochloric acid in a test tube add just one drop of a 0.4 per cent. alcoholic solution of thymolsulfonephthalein. Add the same amount of indicator to (a) 5 c.c. 0.001 N hydrochloric acid, and (b) 5 c.c. 0.1 N lactic acid. Compare the colors. The hydrogen ion concentration of the 0.1 N lactic acid should be less than that of the 0.01 N hydrochloric acid.

8. Colorimetric Determination of Hydrogen Ion Concentration.—The hydrogen ion concentration of most biological fluids is considerably less than in the solutions tested in the preceding experiments, and dilute hydrochloric acid solutions cannot be used here as standards, owing to the ease with which the p^{H} is changed by slight contamination. Instead, it is necessary to have a series of standard buffer mixtures, whose p^{H} is not readily altered.

A suitable set of stock solutions from which to prepare such standards is: 0.2 M monopotassium phosphate, 0.2 M acetic acid, 0.2 M boric acid (containing also 0.2 M potassium chlorid), and 0.2 M sodium hydroxid. The sodium hydroxid solution must be prac-

tically free from carbonate, and should not contain calcium or barium. The compositions of the standard mixtures (diluted to 200 c.c. in each case) are given in the table below. These mixtures, once made up, can be relied upon for only about one week, but the stock solutions from which they are prepared should keep indefinitely in receptacles of resistance glass, except the sodium hydroxid solution, which will gradually increase in strength unless kept in a paraffined bottle.

The determination is carried out as follows: With a pipet transfer two c.c. of the unknown solution to a measuring cylinder and add water to make the total volume 20 c.c.[†] Mix, and add one drop (no more) of phenol red solution. Compare the color with the set of standards.[†] In case the color is beyond the limits for phenol red on either side, repeat with the next indicator in order (see table), until the unknown has been correctly matched against one of the standards. The p^H reading may be made to one-tenth unit by adding or subtracting 0.1 in case the color lies definitely between two consecutive standards.

Determine, in the manner described, the hydrogen ion concentration of two unknowns (supplied). The same method will later be applied to urine.

Indicator: METHYL RED.[*]

					p^H
50 c.c.	0.2 M CH_3COOH	and	23.0 c.c.	0.2 M NaOH	4.6
50 c.c.	"		29.0 c.c.	"	4.8
50 c.c.	"		34.5 c.c.	"	5.0
50 c.c.	"		38.5 c.c.	"	5.2
50 c.c.	"		42.5 c.c.	"	5.4
50 c.c.	"		45.0 c.c.	"	5.6

[*] Dimethylaminoazobenzene-o-carboxylic acid (0.4 per cent. alcoholic solution; use one drop).

Indicator: BROMCRESOL PURPLE.[†]

50 c.c.	0.2 M KH_2PO_4	and	3.7 c.c.	0.2 M NaOH	5.8
50 c.c.	"		5.7 c.c.	"	6.0
50 c.c.	"		8.6 c.c.	"	6.2
50 c.c.	"		12.6 c.c.	"	6.4
50 c.c.	"		17.8 c.c.	"	6.6

[†] Dibromo-o-cresolsulfonephthalein (0.04 per cent. aqueous solution of monosodium salt; use 2 drops).

[*] In this work, pipets should never be blown out, and water should not be taken from a wash bottle that has been blown into, since a small amount of carbon dioxid readily spoils the result.

[†] A series of diluted standard mixtures, with the indicators already added, will keep for a short time, and offers the most convenient arrangement when a great many p^H determinations are being made simultaneously.

Indicator: PHENOL RED.*

50 c.c.	0.2 M KH_2PO_4	and	23.6 c.c.	0.2 M NaOH	6.8
50 c.c.	"		29.6 c.c.	"	7.0
50 c.c.	"		35.0 c.c.	"	7.2
50 c.c.	"		39.5 c.c.	"	7.4
50 c.c.	"		42.8 c.c.	"	7.6
50 c.c.	"		45.2 c.c.	"	7.8
50 c.c.	"		46.8 c.c.	"	8.0

* Phenolsulfonephthalein (0.02 per cent. aqueous solution of monosodium salt; use 3 drops).

Indicator: THYMOL BLUE.†

50 c.c.	0.2 M H_3BO_3,KCl	and	5.9 c.c.	0.2 M NaOH	8.2
50 c.c.	"		8.5 c.c.	"	8.4
50 c.c.	"		12.0 c.c.	"	8.6
50 c.c.	"		16.3 c.c.	"	8.8
50 c.c.	"		21.3 c.c.	"	9.0

† Thymolsulfonephthalein (0.04 per cent aqueous solution of monosodium salt; use 2 drops).

9. Special Test for Hydrochloric Acid.—Gunzberg's reagent (2 g. phloroglucin and 1 g. vanillin in 100 c.c. alcohol) is very reliable as a means of distinguishing between hydrochloric acid and lactic or other organic acids. The reaction is best carried out as follows:

Transfer 5-6 drops of the reagent to a shallow evaporating dish, and evaporate to dryness over a water bath consisting simply of a beaker of boiling water. The alcoholic solution spreads all over the dish, leaving a thin coating of the dry reagent. By means of pipets, or glass tubes drawn out like pipets, transfer one drop of .01 N hydrochloric acid to one side of the dish, and on another side deposit one drop of .1 N lactic acid, and again place the dish on the water bath. A purplish ring is quickly formed around the hydrochloric acid drop while the lactic acid remains colorless.

Heating over the flame may be substituted for the water bath, but the least overheating tends to obscure the reaction by charring. This reaction is extensively used in the examination of stomach contents.

10. Special Test for Lactic Acid.—More or less specific tests for lactic acid are known and are considered important because of the frequency with which lactic acid is found in the stomach contents of those suffering from carcinoma of the stomach. A convenient yet reliable method is the following:

To 5-10 c.c. of .1 N lactic acid (or filtered stomach juice) in a large test tube add a few drops (.5 c.c.) of normal hydrochloric acid and about 10 c.c. of ether. By cautiously inverting the test

tube during 3-4 minutes (taking care to avoid explosions due to expanding ether vapors) the lactic acid is in part taken up by the ether. By means of a 25 c.c. or 50 c.c. pipet and suction, remove the lower aqueous layer as completely as possible. Decant the remaining ether into another test tube so as to free it from the few drops of aqueous solution not taken out by the pipet. Then add to the ether solution .2 per cent. ferric chlorid solution,* a little at a time with shaking, until the maximum yellow color is obtained. The amount of solution added and the depth of the color obtained give a rough index as to the amount of lactic acid present.

Old, deep colored, ferric chlorid solutions frequently fail to give the test for lactic acid. By the addition of hydrochloric acid, in the proportion of 1 c.c. concentrated acid to 5 c.c. of 10 per cent. ferric chlorid solution, the pale color of the latter and its value as a reagent for lactic acid are restored.

1 c.c. of acidified 10 per cent. ferric chlorid solution diluted with 40-50 c.c. of tap water gives a suitable solution for the test.

11. Nitrogen Determination in Ammonium Salts.—The most convenient and useful analysis of nitrogenous products of physiological significance is the determination of the nitrogen. The nitrogen of such products can be split off by hydrolysis in the form of ammonia, which can then be determined by distillation and subsequent titration.

In a small beaker weigh (to the fourth decimal) 3-3.5 g. pure ammonium sulphate. The salt contains traces of water (.5-1 per cent.), unless it has been dried by heating 1-2 hours at about 110° C.; it should be kept in a desiccator over sulphuric acid. Dissolve the salt without the loss of a single crystal in a 500 c.c. volumetric flask, add 1-2 c.c. concentrated hydrochloric acid, and fill up to the mark with water. The acid is added to keep out moulds. Mix thoroughly and transfer to a dry bottle, or to a bottle freshly rinsed twice with about 25 c.c. of the solution.

This solution should be stoppered, labeled, and preserved as a standard solution. It is used to check up the accuracy of ammonia determinations and sulphate determinations, and later for colorimetric nitrogen determinations.

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* 10 c.c. of 10 per cent. ferric chlorid solution in 400-500 c.c. of tap water.

By means of a pipet transfer 25 c.c. of the ammonium sulphate solution to a 300 c.c. Kjeldahl flask. Add with a cylinder 75 c.c. of water, and add also a small pinch of talcum powder (to prevent bumping during the boiling). Put the flask in a clamp so that the bottom is about 1 cm. above the top of a micro burner.

Transfer 25 c.c. of tenth normal hydrochloric acid to a 300 c.c. Florence flask; add water enough to make a volume of about 150 c.c. and add two or three drops of indicator (alizarin red). *The indicator is added at the beginning so that if by any chance the ammonia distilled over is more than enough to neutralize the acid that fact is at once revealed. When this happens add more standard acid* (10 *or* 25 *c.c.*).

Add about 5 c.c. of saturated sodium hydroxid solution to the contents in the Kjeldahl flask and connect immediately by means of a rubber stopper and glass tubes with the receiver containing the acid. Light the burner without delay, to prevent back suction, and boil *vigorously* for not less than 7 minutes, counting from the time the boiling begins. Withdraw the receiver so that the delivery tube is well above the liquid before removing the flame. Cool the receiver in running tap water. Titrate the remaining uncombined acid with tenth normal alkali. From the figures obtained calculate the amount of nitrogen recovered (in milligrams) and compare with the theoretical figure which the amount of ammonium sulphate taken should give.

In calculating the nitrogen from the titration figures, the amount of acid combined with the ammonia can be regarded as a tenth normal nitrogen solution, each cubic centimeter of which accordingly represents 1.4, or more accurately 1.401, milligram nitrogen. Example: 25 c.c. .1 N HCl was the original amount of acid in the receiver. After the distillation the titration of the distillate required 2.1 c.c. of .1 N NaOH. The ammonia had therefore neutralized 25—2.1 or 22.9 c.c. of the tenth normal acid, $22.9 \times 1.4 = 32.06$ (milligram nitrogen).

12. Kjeldahl's Method for Determining Nitrogen.—In a 100 c.c. volumetric flask dissolve 1-1.5 g. accurately weighed urea. Add a few drops (.5-1 c.c.) concentrated hydrochloric acid, and make the volume up to 100 c.c., mix, and transfer to a clean and dry bottle, label, and preserve.

Pipet 5 c.c. of this solution into each of two Kjeldahl flasks,

16.6 cc 5% $CuSO_4$
100 cc 85% Phosphoric
33.3 con H_2SO_4

add 15 c.c. concentrated ammonia free sulphuric acid and 2 c.c. 5 per cent. copper sulphate solution, and boil 30-40 minutes.

When organic substances are boiled with strong sulphuric acid both oxidation and hydrolysis take place. The oxidation occurs at the expense of the oxygen in the sulphuric acid, and the latter is consequently reduced. The sulphurous fumes thus produced are very irritating to the mucous membranes of the nose and throat. The digestion must, therefore, be made in a hood having a reasonably good draft.

Instead of a hood a "fume absorber" can be used. By the help of an ordinary water pump (of glass) the fumes are then partly aspirated directly into the drain pipes, and the remainder is collected in the lower part of the fume absorber.

In Kjeldahl's method sulphuric acid is used for the destructive digestion but other substances are added to hasten the process. These accessory substances act either as catalyzers (copper sulphate or mercury) or, when added in large quantities, they raise the temperature and thus hasten the digestion. Potassium sulphate (5-20 g.) is most commonly used for the purpose of raising the temperature. In the modification here described a mixture of sulphuric acid (1 volume) with phosphoric acid (3 volumes) is substituted for sulphuric acid. This mixture gives a very high temperature, but it acts on glass much more rapidly than sulphuric acid alone and can not therefore be used except in connection with digestions which can be completed in a few minutes. It is probably the best for the destructive digestion of urine. And only 5 c.c. are taken for each digestion instead of 15-20 c.c., the amount required in the case of sulphuric acid.

The acid mixture is prepared as follows: To 50 c.c. of a 5 per cent. copper sulphate solution add 300 c.c. of 85 per cent. phosphoric acid and mix. Add 100 c.c. of concentrated sulphuric acid (free from the least trace of ammonia), mix, and cover well, to prevent absorption of ammonia from the air.

A 10 per cent. solution of ferric chlorid is also required.

When urea is decomposed by means of boiling concentrated sulphuric acid, it is simply hydrolyzed into carbonic acid and ammonia and a solution of ammonia in a very large excess of acid is obtained. The presence of all this acid must be taken into account when preparing to remove the ammonia by distillation. (The amount of alkali required should be determined by a

rough titration of 5 c.c. of the acid dissolved in 500-700 c.c. of tap water, adding the saturated alkali with a measuring cylinder.) A 20 to 30 per cent. excess of alkali should be added for the distillation.

With a pipet transfer 5 c.c. of the urea solution (or urine) to a Kjeldahl flask (cap. 300 c.c.); add 5 c.c. of the phosphoric sulphuric acid mixture and 2 c.c. of ferric chlorid solution; add also 3 or 4 pebbles, to prevent bumping. Fix the flask in a clamp (in a hood) so that the bottom is only about 1 cm. above the top of a micro burner. Boil with a full flame until all the water is driven off and the flask becomes filled with white, dense fumes. At this point cover the mouth of the flask with a watch glass and note the time. Continue the heating (without changing the flame) for two minutes. At the end of two minutes reduce the flame; the white fumes should now be confined within the flask. With the small flame the heating is continued for two minutes, making a total boiling period of four minutes, counting from the time the mouth of the flask was closed. Remove the flame and let cool for not less than four nor more than five minutes and then add, with a cylinder, 50 c.c. of water. If the cooling process is made too long the contents in the flask, now chiefly metaphosphoric acid, solidify and then do not mix well with water.

The ammonia is to be distilled into a receiver. The latter should contain from 25 to 75 c.c. of tenth normal acid, the amount depending on how much ammonia is expected. The receiver should also contain water enough to make a volume of about 150 c.c. Without adding more water to the hot acid solution in the Kjeldahl flask introduce the necessary alkali, usually 15 c.c. of saturated sodium hydroxid, and connect promptly with the receiver. With the bottom of the flask only about 1 cm. from the top of the micro burner, boil vigorously for five minutes, counting from the time the solution begins to boil hard. At the end of five minutes, withdraw the receiver, allowing it first to rinse itself with steam for a few seconds. Beginners can advantageously replace the receiver with another flask or a beaker containing 100 c.c. of water, indicator, and a drop of tenth normal acid, and continue distillation for two or three minutes, so as to be sure that none of the ammonia failed to get into the first receiver.

Cool the first distillate and titrate and compare the nitrogen

value obtained with the theoretical figure which the urea should give.

The process for the determination of the total nitrogen in urine (5 c.c.) is exactly the same as the process described above for urea. In urine there is, however, considerable complex organic matter to be oxidized and some charring and foaming is encountered. With urine it is usually necessary to have not less than 50 c.c. of acid in the receiver to begin with. The total nitrogen is likely to be from 20 to 30 times as much as the ammonia nitrogen found by the permutit or aeration process. (p. 93.)

13. Determination of Nitrogen in Uric Acid.— Transfer 50-70 mg. of pure uric acid to a clean, dry test tube. Weigh the test tube and uric acid (to the fourth decimal). Shake most of the uric acid into a dry Kjeldahl flask, and again weigh accurately the empty test tube. The difference between the two weighings is the amount of uric acid taken. In the same way, charge another dry Kjeldahl flask with 50-60 mg. of uric acid.

To each flask add 5 c.c. of the phosphoric sulphuric acid mixture described in the preceding section. Digest, distil and titrate as in the case of urea. Calculate the absolute and percentage amount of nitrogen and compare with the theoretical figures.

PART II

CATALYSIS, CATALYZERS, FERMENTS

1. **Hydrogen-ion.**—In each of two test tubes place about 5 c.c. of 2 per cent. cane sugar solution. To one add 5 c.c. of half normal hydrochloric acid to the other 5 c.c. of water. Heat both in a beaker of boiling water for ten minutes. Cool. Transfer 5 c.c. of a sugar reagent (alkaline copper solution) to a test tube, add about one-half c.c. of one of the heated cane sugar solutions and boil 1 to 2 minutes. Repeat with the other cane sugar solution.

Cane sugar when split by hydrolysis yields reducing sugars.

2. **Hydroxyl-ion.**—Fill a small test tube up to within about 1 c. from the top with 1 per cent. tannic acid solution. Add a few drops sodic hydrate solution, mix quickly, and let stand for a few minutes.

3. **Metallic salts.**—To 1 c.c. of urine in each of two Kjeldahl flasks add 5 c.c. concentrated sulphuric acid. To one add a crystal of copper sulphate. Heat to gentle boiling for 10 minutes to 20 minutes. Compare the rate of disappearance of the brown color.

4. **Pepsin.**—Prepare a pepsin solution from the mucous membrane of a pig's stomach as follows: Strip off the mucous membrane from a pig's stomach, mix with 300 c.c. of approximately decinormal hydrochloric acid (the "concentrated hydrochloric acid" is approximately a 10 N solution) in a wide-mouth bottle (capacity 900-1,000 c.c.), and let stand over night. Remove by decantation 75 c.c. of the stomach extract. To the remaining mixture in the bottle add 5-10 c.c. of chloroform, cork tightly, shake vigorously for a few seconds, label, and place in an incubator. The stomach will digest itself and give a solution suitable for the later study of peptones.

Suspend a piece of egg albumen, or a Mett tube, in the top of each of the following solutions:

(a) 5 c.c. of undiluted gastric extract,

(b) 5 c.c. consisting of one part of juice to 3 parts .1 N hydrochloric acid,

(c) 5 c.c. of juice diluted as in (b) and heated in a water bath at a temperature of 75° for 15 minutes.

Put all the solutions in an incubator (the warm room) over night. Note the results and explain. Compare results with those of other students.

To 20 c.c. of the pepsin solution add half a volume of disodic phosphate solution (10 per cent.) and half a volume of calcium acetate solution (10 per cent.). Filter off the precipitate and wash once with water. Dissolve the precipitate in a minimum quantity of half normal hydrochloric acid. Dialyze over night. Remove the liquid from the dialyzing tube, test its reaction with congo red, and dilute with water or with dilute HCl (which?) to the volume of the pepsin solution originally taken (20 c.c.). With this solution repeat (a) and (b).

5. Trypsin.—Free a beef pancreas from fat, cut up fine, and weigh; transfer to wide-mouth bottle and add 3 c.c. 10 per cent. alcohol for each gram of pancreas. Add 5 c.c. of chloroform, cork tightly, shake, and set aside for two or three days.

Then take out 25-35 c.c. of the clear liquid and pour on a filter. Stopper tightly again, and put the bottle in the warm room (or incubator) to be preserved for later experiments on "amino acids." With the filtered portion of the pancreatic extract make the following experiment:

Test its digestive power on egg albumen (Mett's tubes).

6. Urease.—1. Heat a water bath from 50 to 55° C. Transfer 35 c.c. of tenth normal hydrochloric acid, 2 drops of alizarin red, and 115 c.c. of water to a 300 c.c. Florence flask.

Transfer 5 c.c. of 2 per cent. urea solution to a 300 c.c. Kjeldahl flask. Add 2 c.c. of neutral phosphate mixture (0.2M primary phosphate and 0.3M secondary phosphate; p^H 7.0). Mix and then add 2 c.c. of 5 per cent. alcoholic Jack bean extract (p. 107); stopper tightly; place the flask in water bath at 50 to 55° and shake gently for 1 or 2 minutes to promote speedy warming. Continue the urease digestion for exactly 10 minutes.

Cool the flask to promote condensation of ammonia vapors; then add 50 c.c. of water, 5 to 7 drops of paraffin oil (to prevent foaming) and 2 g. of borax. Connect at once with the receiver, as in nitrogen determinations; distil for 6 to 8 minutes. Cool and titrate.

Calculate how much of the urea was decomposed.

2. Repeat the experiment described under 1 but substitute a water-bath temperature of 75-80° C.

3. Repeat at room temperature, 15 to 20° C.

4. Repeat at room temperature but substitute 2 c.c. of acid phosphate (0.5M primary phosphate and 0.006M secondary phosphate; p^H 5.0) for the neutral phosphate.

5. Repeat with 2 c.c. of alkaline phosphate (0.004M primary phosphate and 0.5M secondary phosphate; p^H 9.0).

6. Add 2 c.c. of mercuric chlorid solution or of Nessler's reagent to a previously rinsed Kjeldahl flask. Shake for one or two minutes. Pour out the mercury solution and rinse the flask three or four times with water. With the apparently clean flask repeat either 1 or 3. Very small traces of mercury destroy urease as well as many other *enzymes*.

7. Reversible Reactions (Mass Law).—Mix 5 c.c. methyl acetate, 100 c.c. water, and 1 drop concentrated sulphuric acid in a small flask (200-300 c.c.).

(a) Titrate 5 c.c.

(b) Boil for about 5 minutes, using reflux condenser. Cool, remove 5 c.c., titrate the acidity (what indicator?), and calculate the acidity for 100 c.c.

Continue the boiling for one hour, and repeat the titration.

(c) Mix 5 c.c. of glacial acetic acid with 100 c.c. of methyl alcohol, and add one drop of concentrated sulphuric acid. Remove 5 c.c., dilute this with water, and titrate the acidity. Introduce the preparation into a 250 c.c. flask attached to a reflux condenser, and boil for two hours. Cool, remove 5 c.c., dilute, and titrate as before.

PART III

FATS

1. Solubility of Fats.—Test the solubility of tallow in water, 5 per cent. NaOH, ether, chloroform, and alcohol, carefully avoiding the vicinity of a flame.

Let a drop of the ether solution fall on paper, and note the result.

Dissolve in 3 c.c. of warm benzene enough tallow to give a moderate precipitate on cooling. Place some of the precipitate on a slide under a cover glass, examine, and describe. Note especially the shape of the ends of the individual crystals.

2. "Iodin Number." Degree of Unsaturation of Fats; Wys' Method.—Start simultaneously the determination of the iodin number of cottonseed oil and beef tallow. Weigh about .3 g. of cottonseed oil, or about 1 g. of beef tallow, into a 250 c.c. flask, and dissolve in chloroform (10 c.c.). Add 25 c.c. Wys' iodin solution with a pipet, stopper, and put in a dark place for half an hour. Add 15 c.c. of 10 per cent. potassium iodid, and dilute with 100 c.c. of water, titrate the excess of iodin (partly in solution in the water, partly in the chloroform) with .1 N sodium thiosulphate, by running the latter into the flask until, after repeated shaking, both the chloroform and the watery solution are but faintly straw colored. Then add a few drops of a 1 per cent. starch solution, and continue the titration to the disappearance of the blue color.

While waiting for absorption to take place, the value of the iodin solution may be determined in terms of .1 N thiosulphate by adding KI and titrating in the same way as above. The difference between the two valves represents the amount of iodin absorbed by the fat, and is calculated in grams of iodin per 100 g. of fat.

Example:

.3 g. cottonseed oil, when treated as above, required 35 c.c. of .1 N thiosulphate for back titration.

25 c.c. of the iodin solution required 60 c.c. of .1 N thiosulphate.

The oil therefore absorbed iodin corresponding to $60 - 35 =$ 25 c.c. .1 N thiosulphate, i.e., 25 c.c. .1 N iodin or $25 \times .0127$ g. Iodin = .317 g. I. The Iodin Number is, therefore, $\frac{100}{.3} \times .317 = 105.6$.

WYS' IODIN SOLUTION.—Dissolve 13 g. of iodin in 1 liter of glacial acetic acid. Titrate the iodin content of the solution, and then pass washed and dried chlorin gas into the solution until the titration number is doubled. A very distinct change in the color of the solution indicates when this has taken place.

The thiosulphate solution is prepared by dissolving 24 g. of the crystallized salt in 1 liter of water and standardizing it in the usual way (*see* page 153).

3. Saponification and Preparation of Fatty Acids.—Heat about 20 g. of beef tallow with 100 c.c. of alcoholic solution of sodium hydrate on a water bath over night, or until the residue is dry. To the mixture add about 300 c.c. water and heat to boiling. To the hot solution add a few drops of methyl orange; while continuing the heating (and stirring), acidify with dilute sulphuric acid, and filter. Save the filtrate which contains glycerin, then wash the fatty acid residue several times with hot water. Throw away the washings.

Transfer the "glycerol filtrate" to an evaporating dish, label, and place on the water bath for evaporation of dryness.

4. Solubility of Fatty Acids.—Test the solubility of the fatty acid mixture prepared from tallow in water, 5 per cent. NaOH, ether, alcohol, and benzol. Compare the results with those obtained with fat.

Let a drop of the ether solution fall on paper, and note the result.

Dissolve enough of the fatty acid mixture in warm alcohol to give moderate precipitate on cooling. Examine the crystals under the microscope, and describe as in the case of the fat crystals.

5. Spontaneous Saponification of Fats.—Dissolve about .5 g. tallow, or a few drops of oil, in 10 c.c. warm alcohol in a test tube (avoid fire!). Add 3-4 drops phenolphthalein solution, and

then, drop by drop, tenth normal sodic hydrate solution (alcoholic sodic hydrate solution is best) until the indicator reveals a distinctly alkaline reaction. Let the mixture stand in a warm room over night, and again add alkali (drop by drop) until the alkaline reaction reappears.

6. **Titration of Higher Fatty Acids.**—Dissolve about .2 g. fatty acid mixture in 10 c.c. warm alcohol or benzol (avoid fire!). Add 3-4 drops phenolphthalein, and titrate with tenth normal alcoholic sodic hydrate solution until an alkaline reaction is obtained. One cubic centimeter of the alkali corresponds to how much fatty acid?

7. **Fat digestion with Lipase (Castor Bean).** — Remove the shells from 10 g. fresh castor beans, break them up as fine as possible, and allow to stand over night in a loosely stoppered test tube full of alcohol ether mixture. Pour off, grind the beans to a powder in a small mortar, transfer to a test tube, and let stand under ether over night. Filter with suction, and wash two or three times with small amounts of the alcohol ether mixture. Grind with the powder in the order named, 5 c.c. .1 N sulphuric acid (supplied), 5 c.c. of neutral cotton oil (Sp. gr. .92), and 5 c.c. lukewarm water. The water should be added a little at a time and thoroughly worked into the mixture so that at the end of the operation a good emulsion is secured. Cover the evaporating dish, and let stand in a warm place over night.

Add 50 c.c. of alcohol, 10 c.c. of ether, and a few drops of phenolphthalein, and titrate with .5 N sodium hydrate. Calculate the amount of fatty acid and the per cent. of fat digested.

8. **Glycerol and the Acrolein Test.**—To about 5 g. acid potassium sulphate ($KHSO_4$) in a porcelain crucible add one drop of glycerin, heat over a direct flame, and note the pungent odor and tear-begetting quality of the fumes. Note how much heat must be applied to secure an unmistakable test.

When the filtrate (saved from the saponification of beef tallow) has evaporated to dryness, the residue obtained is sodium sulphate; mixed with it there should be some glycerin. (How much glycerin might be there?)

Mix with a glass rod 2-3 g. of this residue with 5-6 drops concentrated sulphuric acid in a dry crucible, and apply heat. If

an unmistakable acrolein test is not obtained, repeat with more of the residue.

9. **Emulsification.**—Put 1-2 c.c. of a solution of sodium carbonate (.2 per cent.) in a watch glass, and place in the center a drop of rancid oil. The oil soon shows a white rim, and a milky opacity spreads over the solution. Note with the microscope the active movements in the vicinity of the fat drop, due to the separation of minute particles of oil.

Examine a sample of milk under the microscope. The fat should be in a state of fine emulsion.

10. **Lecithin.**—Demonstrate myeline movements (observed on mixing lecithin with water).

Mix a small piece of lecithin in water in a test tube. Shake vigorously for a time, and state what occurs. To the contents add concentrated caustic soda and boil. Note the fishy odor of trimethylamine (from the cholin). Acidify the solution—fatty acids are precipitated, and glycerophosphoric acid is left in solution. Write the graphic formula for lecithin.

11. **Cholesterol; Liebermann's Reaction.**—Dissolve a crystal of cholesterin in 10 c.c. of dry chloroform, and to this solution add a few drops of acetic anhydrid (formula?) and one drop of concentrated H_2SO_4. Shake. The liquid becomes rose red, blue, then dark green.

12. **Problem.**—On the basis of the solubilities and reactions of fats, fatty acids and soaps, work out a scheme for their separation and identification. Apply the scheme to two unknowns furnished. Hand in a dated and signed report on the same.

PART IV

CARBOHYDRATES

The numerous very old methods of testing for sugar, including Trommer's and Fehling's, are now only of historical interest, except perhaps in connection with some State Board Examinations. They are omitted here.

1. Benedict's Test.—One of the best qualitative tests for sugar in urine by means of copper solutions is the one recently proposed by S. R. Benedict. Benedict's reagent is so adjusted that it is rather more sensitive to dextrose than Fehling's solution, yet is not reduced by creatinin or uric acid, and little, if at all, by chloroform (which is often added as a preservative to urine). Unlike Fehling's reagent it consists of a single solution. The reagent is made as follows:

Dissolve 85 g. sodium citrate and 50 g. anhydrous sodic carbonate in 400 c.c. of water. Dissolve 8.5 g. copper sulphate in 50 c.c. of hot water. Pour the copper sulphate solution slowly, and with stirring, into the alkaline citrate solution. Filter if necessary. Label and preserve.

Heat to boiling about 5 c.c. of Benedict's reagent in a test tube together with a pebble or two, to prevent bumping. Add about 8 drops of sugar solution (or urine) and boil for 2 minutes. If more than two- or three-tenths per cent. of sugar is present, the solution will be filled with a colloidal (greenish, yellow, or reddish) precipitate. With smaller amounts of sugar the precipitate will usually appear only on cooling (the cooling should not be hastened by immersion in cold water).

2. Folin-McEllroy's Test for Sugar.—The reagent in this test is made as follows: Dissolve 100 g. of sodic pyrophosphate, 30 g. of disodic phosphate and 50 g. of dry sodium carbonate in approximately 1 liter of water by the aid of a little heat. Dis-

solve separately 13 g. of copper sulphate in about 200 c.c. of water. Pour the copper sulphate solution into the phosphate-carbonate solution and shake.

To 5 c.c. of the solution in a test tube add 5-8 drops of urine (never add more than 0.5 c.c.) and boil for 1-2 minutes or heat in a beaker of boiling water for 3 minutes. If more than the normal traces of sugar be present the hot solution will be filled with a colloidal (greenish-yellow or reddish) precipitate as in Benedict's test. This test is a trifle more sensitive than Benedict's, therefore when working with urine only a distinctly positive test obtained with the solution still *hot* is to be regarded as positive.

3. Reduction Test for Sugar in Normal Urine. — That most human urines contain distinct traces of reducing sugar can be shown by the use of more sensitive copper reagents. One such reagent can be made as follows:

(A) Dissolve 5 g. of crystallized copper sulphate in 100 c.c. of hot water, and to the cooled solution add 60-70 c.c. of pure glycerin.

(B) Dissolve 125 g. of anhydrous potassium carbonate (with stirring) in 400 c.c. water.

Mix one volume of the glycerin-copper solution (A) with two volumes of the potassium carbonate solution (B). Only small portions should be mixed at a time, as the reagent (after mixing) does not keep, but undergoes gradual reduction.

The test for sugar in normal urine is made as follows:

First transfer 5-10 c.c. of the mixed reagent to a test tube, and boil the reagent in order to determine the extent, if any, to which the reagent is spontaneously reduced.

Transfer 1-2 g. picric acid to a small bottle; to it add some of the urine (10-50 c.c.) to be tested, insert a cork, shake for five minutes, and filter. By this treatment the disturbing creatinin and uric acid are removed. Now heat 5-10 c.c. of the copper solution to boiling in a fairly wide test tube (with a pebble to prevent bumping), add 1-2 c.c. of the filtered urine, and boil for 60-75 seconds. If the sugar present is relatively large in amount, the solution becomes turbid while still boiling, as in Benedict's test. If no such turbidity is observed, centrifuge at once, i.e., before cooling. The bottom of the centrifuge will be covered

with cuprous oxid. The crystals to be observed in the supernatant cooling liquid are potassium picrate.

Repeat this test with 1-2 c.c. urine which has not been treated with picric acid, and note how much more abundant the reduction is with ordinary creatinin containing urine.

4. **Phenylhydrazin Test (Osazone Test).**—To 5-10 c.c. of .2 per cent. glucose solution in a test tube add 5 c.c. of a phenylhydrazin solution (containing 5 per cent. phenylhydrazin hydrochlorid, 20 per cent. sodic acetate, and 10 per cent. acetic acid). Heat in a beaker of boiling water for half an hour. Let the test tube remain in the beaker until the water has cooled, and examine the glucose osazone crystals under the microscope.

Write the reaction involved in the formation of osazone.

5. **Glucose Reactions versus other Carbohydrates.** — Apply one of the copper reducing tests, and the phenlyhydrazin test to .2 per cent. solutions of arabinose, levulose, cane sugar, maltose, and lactose.

6. **Selivanoff's Test for Ketose-Sugars.** — Selivanoff's reagent contains .05 per cent. of resorcin and about 12 per cent. of hydrochloric acid.

To 5-10 c.c. of the reagent in a test tube add about 1 c.c. of .1 per cent. solution of levulose, boil for one minute, set aside to cool, and note the development of the color.

Repeat the test with dextrose and with cane sugar (and, if desirable, with other dilute sugar or carbohydrate solutions). Tabulate the results.

7. **Test for Ketose Sugars (Levulose?) in Urine.** — Collect urine for one hour (preferably just before the noon hour). Then take 50 g. of cane sugar and collect urine for another hour. Dilute the smaller volume of urine to that of the larger, or both to a convenient small volume. To 10 c.c. of each in a test tube add 5 c.c. of 10 per cent. lead acetate solution, shake, and filter. Apply Selivanoff's levulose test to about 2 c.c. of each filtrate. Record the results obtained.

Minute traces of levulose in human urine are probably not so infrequent an occurrence as is generally assumed to be the case. The color obtained with urine is, however, not exactly like that

given by aqueous levulose solutions. If a distinct "levulose' output from the cane sugar is obtained, test both urines for reducing sugar, and note whether there appears to be an increase of sugar in the urine of the second period.

8. **Orcin Test for Pentoses.**—The pentose reagent is made by dissolving 1 g. of orcin in 500 c.c. 30 per cent. hydrochloric acid (6:1) and adding 1 c.c. of 10 per cent. ferric chlorid solution.

Heat 5 c.c. of the orcin solution to boiling in a test tube. Remove from the flame, and add (immediately) 1 c.c. 1 per cent. solution of arabinose.

Repeat, adding 1 c.c. .2 per cent. arabinose solution.

Note the color (violet, blue, red, green) and the formation of a precipitate.

Repeat with 1 per cent. arabinose solution previously diluted (a) with 4 volumes of urine (b) with 4 volumes 1 per cent. glucose solution.

Repeat (a) with urine alone (b) with 1 per cent. glucose solution (c) with 1 per cent. cane sugar solution.

9. **Fermentation Test for Sugar.** — Certain sugars (which?) are decomposed by yeast into carbon dioxid and alcohol. The formation of CO_2 in fermentation has been extensively used both for qualitative studies and for quantitative determinations of sugar. Except among physicians who have not the facilities for making other tests, the fermentation method is now seldom used.

To some .5 per cent. sugar solution (dextrose, pentose, cane sugar, lactose) in a test tube add a small piece of yeast. Shake to make uniform mixtures, and with each fill a "fermentation tube." Substitute water for sugar solution in a control test, and set the tubes aside in a warm place over night. Record the results.

10. **Benedict's Method for the Determination of Sugar.**—Prepare 500 c.c. of Benedict's solution as follows: Dissolve 9 g. pure copper sulphate in a 500 c.c. volumetric flask with about 100 c.c. distilled water. Dissolve 50 g. anhydrous sodic carbonate, 100 g. sodium citrate, and 50 g. sodium sulphocyanate in 250 c.c. distilled water. The copper sulphate must be weighed accurately on the analytical balance. Pour the copper solution,

slowly, with stirring and without loss of a single drop, into the alkaline citrate solution. Then pour the mixed solution back into the measuring flask without loss, add 5 c.c. 5 per cent. potassium ferrocyanid solution, and with the rinsings make the total volume up to 500 c.c. Mix, transfer to a clean, dry bottle, label, and preserve. Twenty-five cubic centimeters of the solution corresponds to 50 mg. of dextrose, 52 mg. of levulose, 67 of lactose, or 74 of maltose.

The determination is carried out as follows:

Measure 25 c.c. of Benedict's solution into a porcelain dish, add 5-10 g. of solid sodic carbonate, heat to boiling, and while boiling run in the sugar solution (or urine) fairly rapidly until a white precipitate begins to form. Then add the solution more slowly (with slower boiling) until the last trace of the blue color disappears. The addition of the sugar solution should be done at such a speed that the boiling solution is kept nearly constant in volume during the operation. The original sugar solution (or urine), if concentrated, should be diluted so that not less than 10 c.c. will be required to give the amount of sugar which the 25 c.c. of reagent is capable of oxidizing.

Five divided by the volume of sugar solution taken gives the per cent. of sugar. Check the value of the reagent by determining the sugar in .5 per cent. dextrose solution.

11. New Method for Titration of Sugar. — (*See Journ. Biol. Chem.*, 33, 513, 1918; 38, 287, 1919.)

1. Alkaline phosphate mixture. Powder in a large mortar 200 g. of crystallized disodic phosphate ($HNa_2PO_{41}12H_2O$), sprinkle over it about 50 g. of sodium thiocyanate (or 60 g. of potassium thiocyanate). Mix with mortar and spoon for about ten minutes. A uniform semi-liquid paste is obtained. To this paste add 120 g. of monohydrated sodium carbonate (or 100 to 110 g. anhydrous carbonate) and mix with mortar and spoon until a rather fluffy granular powder is obtained. Leave in mortar covered with paper over night, then mix once more. This reagent keeps indefinitely but should be kept in stoppered bottles so as not to lose too much moisture.

2. A saturated solution of sodium carbonate containing 14 to 20 per cent. Na_2CO_3.

3. A copper sulphate solution containing 59 g. of $CuSO_4$, $5H_2O$ and 2 c.c. of concentrated sulphuric acid per liter. The

sulphuric acid in this reagent is added only to prevent precipitation of copper hydrate by the traces of alkali gradually given off by glass. The solution keeps indefinitely.

5 c.c. of the copper solution is reduced by 25 mg. of glucose or levulose, by 40.4 mg. of anhydrous lactose, or by 45 mg. of anhydrous maltose. The normal reduction time is 5 minutes, except in the case of levulose which is reduced within 2 minutes. In working with lactose it should be noted that crystallized lactose contains 1 molecule of water of crystallization.

The titration is best made in large hard glass test tubes. Urines or sugar solutions up to a concentration of 7 or 8 per cent. are titrated directly, that is without any preliminary dilution.

A special sugar buret, total capacity 5 c.c., divided in 0.02 c.c. is used for measuring. This buret should have an accessory tip very fine and about 5 cm. long; it should also have a rubber tube attachment above for filling by suction.

Transfer 5 c.c. of the copper solution to the large test tube; add 1 c.c. of sodic carbonate solution, thereby precipitating the copper and rendering the solution alkaline. Add 5 g. (not less than 4.5 nor more than 5.5 g.) of the solid phosphate mixture. Heat gently, with shaking, until all the salts except for a few isolated particles of sodium carbonate have dissolved. A practically clear solution is usually obtained in less than 1 minute and temperature need not exceed 60° C. Use only a micro burner as the source of heat.

From the sugar buret, filled by suction, with the urine or sugar solution, add 0.4 c.c. to 1.0 c.c. to the warm clear copper solution. With watch in hand (or clearly visible) heat the mixture rapidly to unmistakable boiling. Note, on the second hand, when the boiling begins; from that moment keep track of the time, and thereafter heat only enough to keep the contents just to boiling—by moving the test tube back and forth, through the flame. When bumping begins add a pebble to promote even, gentle boiling.

If the contents of the test-tube do not suddenly become turbid from precipitated cuprous sulphocyanate within the first 15 seconds of boiling, then less than one-half the required amount of sugar has been added and more should be introduced at once. When the full amount of sugar (25 mg.) is present the turbidity appears within 5 seconds after the boiling has begun. The boiling should normally be continued for 3 minutes, counting from

the time that the boiling point was reached, before any more sugar is added. Boil 1 minute after each subsequent addition of sugar. The total boiling period for a correct titration must not be less than 4 or more than 6 to 7 minutes. But the preliminary titration may last for 8 to 9 minutes and if the boiling process has been gentle the result will then be only about 1 per cent. too high.

In the preliminary titration it will frequently happen that the first sugar addition contains more than 25 mg. of glucose, and the greater the excess the more quickly will decolorization of the copper take place.

Time of boiling for complete reduction of copper solution by an excess of glucose.

Glucose	Boiling Time	
Mg.	Min.	Sec.
50	0	25
40	0	40
35	0	55
30	1	20 to 30
27.5	1	30 to 55
25.5	3	

By noting the boiling time in which complete reduction has occurred a valuable guide to the amount of sugar solution to be taken for the next titration is obtained.

After some experience has been gained it should very seldom be necessary to make more than two titrations, a preliminary and a final, for any one sugar determination.

Calculation: 0.025 times 100, or 2.5, divided by the titration figure in c.c., whether this be several c.c. or a fraction of 1 c.c., gives the per cent. of glucose present.

Prepare 100 c.c. of 1, 1.5 or 2 per cent. solution of pure glucose and learn the titration on the basis of that solution. Without some preservative moulds are apt to develop in this solution in the course of 3 or 4 days. It is not a bad plan to substitute tenth normal hydrochloric acid or saturated benzoic acid solution for one-half of the water used in making the solution. Preservatives such as chloroform or toluol, are not very satisfactory in this case because they contaminate the buret so that drops of sugar solution soon begin to stick to the sides.

12. Polariscope Method for the Determination of Sugar.—The specific rotation of a substance is the angle through which the plane of polarized light is turned by 1 dm. of a solution containing 1 g. of the optically active substance per c.c.

A definite temperature and light of a definite wave length (sodium light) must be used in determining specific rotations.

The angle of rotation is determined by means of some form of "polariscope" (polarimeter, saccharimeter, etc.); and the specific rotation is calculated according to the following formula:

$$\text{Specific Rotation} = \frac{\text{Observed Rotation} \times 100}{\text{Percentage} \times \text{Length of Observation Tube (dm.)}}$$

If the specific rotation is known, as in the case of the common sugars, the per cent. of sugar is obtained by the following transposition of the above formula:

$$\text{Percentage} = \frac{\text{Observed Rotation} \times 100}{\text{Specific Rotation} \times \text{Length of Tube (dm.)}}$$

Following are the specific rotations (yellow light) of some common sugars:

Glucose, 52.8; Fructose, —93; Cane Sugar, 66.5; Lactose, 55; Maltose, 137.

The determination of sugar by means of the polariscope is as follows:

Rinse the polariscope tube (length usually 1 or 2 dm.) with the sugar solution, and fill almost to overflowing. Place the glass plate over the open end in such a way that the tube does not contain any air bubble, and screw on the cap. Place the tube in the groove of the polariscope. Light the lamp, and move the eyepiece back and forth until the lines which divide the field are sharp. Then turn the screw until the several divisions of the field are equally illuminated, and take a reading by means of the vernier. The circle upon the disk of the apparatus is divided into quarter degrees; 24 divisions of the vernier correspond in length to .25°. Consequently every division of the vernier corresponds to .01°. Ascertain whether the disk, starting from its middle point, has been moved to the right or to the left of the zero point of the vernier. Read off the number of whole degrees and hundredths. Take several readings by moving the

lever and coming back again to the point where the different parts of the field are equal. Correct for the zero point of the instrument by taking readings with the tube filled with water. This value is added to, or subtracted from, the reading found with the sugar solution.

If a saccharimeter is used instead of the general circular polariscope, the reading on the scale is converted into angular degrees of rotation by multiplying by the factor .345. The percentage of sugar in the solution is then calculated by using the formula given above.

13. Cane Sugar.—With the help of the balance prepare 100 c.c. of a known cane sugar solution (8 to 12 per cent.) So-called lump sugar is better for this purpose than granulated sugar, because of freedom from dust. Determine the concentration of the solution with the polariscope according to the directions given in the preceding section.

Transform a part of the solution into "invert Sugar" and determine the sugar content by titration. The inversion is made as follows:

Transfer 10 c.c. of the cane sugar solution to a 100 c.c. volumetric flask and add 1 c.c. of concentrated hydrochloric acid. Add no extra water. Heat a large beaker or porcelain dish to 70° C. When the water has reached 70° C., immerse the flask in water and, without allowing the temperature of the water bath to sink below 70°, rotate and shake the flask continuously, but gently, for 10 minutes. At the end of this time cool and dilute to the 100 c.c. mark with water and mix.

Instead of heating to 70° complete inversion can also be accomplished by allowing the mixture of sugar and acid to stand at room temperatures over night. The over night process is of course inapplicable in "practical examinations."

In connection with titrations of invert sugar due note must be taken of the 5 per cent. increase in the weight of the sugar accompanying the inversion. In titrating unknown solutions of invert sugar it must not be forgotten that one-half of the sugar is levulose and that levulose reduces alkaline copper solutions much more rapidly than does dextrose. Turbidity within the first 5 seconds of boiling does therefore not necessarily indicate that almost enough or too much sugar has been introduced.

14. Problems.—Determine by titration and by the polariscope the sugar concentration of one unknown solution of glucose, one of invert sugar, and one of lactose. Determine, without the polariscope, the glucose and cane sugar content of one unknown mixture of these two sugars.

Hand in dated, signed reports on these unknowns; each report to represent two unknowns.

15. Preparation of Maltose.—Mix 10 g. of starch with 30 c.c. of cold water until a smooth paste is obtained. Pour this slowly, and with stirring, into 250 c.c. of boiling water in a large beaker, continue the boiling 1-2 minutes, let cool to 75° C., stir in 1 teaspoonful of malt, and keep at this temperature until the mixture becomes thin and watery. Heat again to boiling with stirring, cool to 75°, add another teaspoonful of ferment, and keep at this temperature until a sample no longer gives any color with iodin solution (about two hours). Boil, cool, transfer to a 250 c.c. volumetric flask, make up to volume. Determine the maltose by titration in a filtered sample and calculate the amount of maltose obtained. Taste the solution.

16. Preparation of Milk Sugar.—To 300 c.c. of skimmed milk diluted with 800 c.c. of water, add cautiously 2 per cent. acetic acid to precipitate the casein. When enough has been added the liquid is nearly clear. Filter. Boil the filtrate, and filter off the coagulated albumin. Evaporate the filtrate on the water bath to a thin syrup, and allow it to stand until the sugar has crystallized out.

A more expensive but otherwise much better method for preparing crystallized lactose from milk is as follows: Transfer 100 c.c. of skim milk to a 200 c.c. flask or bottle, add 5 g. of solid picric acid. Shake for 10 minutes, let stand for half an hour and filter into a 300 c.c. flask or bottle. Add to the filtrate 200 c.c. of acetone and let stand for 24 or better for 48 hours. Examine and test the crystalline deposit. It is all milk sugar.

17. Starch and Dextrin.—Heat 300 c.c. of water to boiling in a beaker. Transfer 2 g. of finely powdered starch to a test tube, add 5 c.c. of cold water, shake well and pour the resulting starch suspension, a little at a time, into the boiling water. Boil gently for about 10 minutes. Transfer starch solution to a flask and cool.

Prepare also about 50 c.c. of 2 per cent. dextrin solution, using heat if necessary to get a clear solution.

Commercial dextrin sometimes contains unaltered starch and usually contains reducing sugar. If appreciable traces of starch are present the gradual addition of diluted iodin solution gives at one stage a pure blue color. If no starch is present only red, brown or violet colors appear.

To each of 2 test tubes add 5 c.c. of water. To one add about 2 c.c. of starch solution and to the other about 2 c.c. of dextrin solution. Add diluted iodin solution drop by drop (by means of a pipet) to each test tube until the maximum color is obtained. Note the color obtained in each case. Heat each solution gradually until the colors have disappeared and cool. Heat each test tube again, this time to distinct boiling, and cool. The colored iodin compounds are not very stable at higher temperatures.

Dilute 5 c.c. of the starch solution to 100 c.c. Add varying amounts of this diluted starch solution to 2 c.c. of dextrin solution and apply the iodin test. Note at which stage of iodin addition that the pure blue color is obtained. Note also that a positive starch reaction may easily be missed by adding too much iodin. The iodin solution should be very dilute; the ordinary 1 per cent. solutions need be diluted 20 times. If the original dextrin solution is free from starch, what is the minimum quantity of added starch required to give a positive pure blue reaction?

To 5 c.c. of starch solution add about 10 c.c. of saturated ammonium sulphate solution and shake. Note that starch is precipitated. Repeat with the dextrin solution, if the dextrin is free from starch according to the iodin test, it will not give a precipiate with ammonium sulphate solution. The starch precipitation is not strictly quantitative; a faint starch reaction can usually be obtained with the ammonium sulphate filtrate from starch solutions. Try this (a) with starch filtrates (b) with mixtures of starch and dextrin. A fairly satisfactory separation of starch from dextrin is attained by the help of ammonium sulphate.

Test for reducing sugar in the solutions of starch and dextrin.

Mix 2 to 5 g. of brown crackers with about 50 c.c. of cold water. Filter. Test the filtrate for starch, dextrin, and reducing sugar.

To 5 c.c. of saliva in a small flask add 150 c.c. of the starch solution. (Fresh starch solutions are best for this experiment.) Heat in a beaker of warm water (40 to 42° C.). Test at the end

of 3 to 5 minutes for reducing sugar and repeat until a positive test is obtained.

Continue the digestion at 40 to 42° C. and at 10 minute intervals transfer 5 c.c. portions to each of 2 test tubes. To one add just enough iodin to give an unmistakable color; to the other add enough iodin to give the maximum color. Save the series of test tubes for comparison and note the gradual disappearance of the starch and the formation and disappearance of dextrin.

18. Glycogen.—Cut four fresh (Why fresh?) oysters into small pieces, and throw into four times their weight of boiling water slightly acidulated with acetic acid. After boiling for a short time, remove the pieces, grind in a mortar with some sand, return to the water, and continue the boiling for several minutes. Filter while hot. The opalescent solution thus obtained is an aqueous solution of glycogen and other substances.

With the solution of glycogen thus obtained, make the following tests:

Add iodin solution drop by drop to a portion of the glycogen solution. The liquid will assume a dark red color. This color disappears, with the exception of the color due to the iodin, upon gentle heating, and reappears upon cooling. (Compare with dextrin.)

Test the glycogen solution with Benedict's solution and note the result.

Add some saliva to a portion of the glycogen solution, and put in the warm room until the next day. Remove and divide into two portions. Test one with iodin solution for glycogen, the other for sugar. Report the result.

PART V

PROTEINS

A fundamentally important general consideration to be noted in the study of protein materials is that proteins are colloids, and that colloidal solutions differ materially in their physical and chemical reactions from the corresponding reactions of ordinary true solutions (of crystalloids).*

1. Dialysis of Colloidal and Molecular Solutions.—In a parchment dialyzing tube place 15 c.c. of blood serum (protein) and 1 c.c. of strong salt solution, Na_2SO_4. The protein is present in colloidal form, the salts in molecular (ionic) solution. Suspend the tube in a 500 c.c. beaker of distilled water, and let the whole stand over night. Test a portion of the dialysate for protein by boiling in a test tube with one drop of dilute acetic acid. Has protein passed through the membrane? Test the dialysate for chlorids and sulphates. Do salts dialyze through parchment membranes?

2. Suspension Colloids; Suspensoids. PREPARATION.—Prepare a colloidal solution of gum mastic as follows: Drop from a buret 1 c.c. of saturated alcoholic solution of gum mastic, slowly and with stirring, into 100 c.c. of distilled water. Filter.

ACTION OF ELECTROLYTES.—To 10 c.c. portions of the above filtrate add

1. 5 c.c. tenth normal HCl,
2. 5 c.c. tenth normal NaOH,
3. 5 c.c. normal NaCl,
4. 5 c.c. urea solution,
5. 5 c.c. concentrated cane sugar solution.

Let stand, note what occurs, and explain.

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* Fats and the more complex carbohydrates behave also as colloids; so indeed do all substances which are insoluble in water, when suspended in water in a state of sufficiently fine division. Colloidal solutions of metallic platinum (one of the least soluble of metals) are frequently employed in the study of certain ferment reactions. Proteins occur in nature almost wholly in the form of colloidal solutions.

Effect of Heat.—Boil a portion of the solution in a test tube.

Evaporate 10 c.c. of solution to dryness on the water bath, and try to redissolve it.

3. Emulsion Colloids; Emulsoids.—Preparation.—To 5 g. gelatin in a beaker add 60 c.c. of water, and heat gently with constant stirring until the gelatin is all dissolved.

Action of Electrolytes.—To 10 c.c. portions of the gelatin solution add,

1. A few drops 10 per cent. NaCl,
2. A few drops concentrated HCl.

Evaporate 10 c. c. to dryness on the water bath, and try to redissolve.

Reversibility of Coagulation by Electrolytes.—To 25 c.c. of a protein solution add solid ammonium sulphate with shaking until the solution is saturated, and note what happens. Filter and press the precipitate as dry as possible between layers of filter paper. Try to redissolve the precipitate in water.

Reversibility of Coagulation by Heat.—To another 25 c.c. portion of the same solution add 2-3 drops dilute acetic acid. Heat to boiling. Filter, press the precipitate dry as before, and try to redissolve in water. Tabulate the differences between emulsoids and suspensoids.

4. "Hydrophile" Colloids.*—To some gelatin in a test tube add just sufficient water to cover it. Let stand and note changes.

5. Reversibility of "Sol" and "Gel" States—Gelatinization as a Special Case of Coagulation.—Warm the above mixture until solution is complete. Cool under the cold water tap. What occurs? Repeat the heating and cooling a number of times.

6. Coagulation by Oppositively Charged Colloids.—To 10 c.c. colloidal arsenic solution add 10 c.c. colloidal $Fe(OH)_3$. Let stand and note what occurs.

7. Rate of Diffusion of Molecular and Colloidal Solutions.—Melt some 4 per cent. gelatin or 2 per cent. agar jelly in a beaker by standing in hot water. Fill 5 small test tubes ⅓ full and allow to solidify. To the jelly tubes, 1-5 respectively, add an equal volume of

1. Colloidal As_2S_3,
2. Colloidal $Fe(OH)_3$,

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* "Hydrophile" colloids are one class of emulsoids—not all emulsoids are "hydrophilous."

3. Picric acid,
4. Copper sulphate,
5. Congo red.

Allow to stand 2 days. Compare the speed of diffusion, i.e., the distance which the various substances have penetrated the jelly. What is the relation between the size of particles and rate of diffusion?

1. Test for Nitrogen, Sulphur, and Phosphorus in Protein.— Put a little dry protein * into a dry, cheap test tube. Add a piece of metallic sodium the size of a pea, and heat strongly for a few minutes. Cool. Carefully and without handling the material, break into a dry evaporating dish. Cover the substance with a wet filter paper. After five minutes cautiously add 25 c.c. water. Stir well. Filter into a test tube.

(a) To 5 c.c. add a few drops of ferrous sulphate and a drop of ferric chlorid solution. Warm and acidify with concentrated hydrochloric acid, noting the result.

(b) Acidify 5 c.c. with nitric acid and add a few drops of ammonium molybdate solution. Let stand over night and look for a yellow crystalline precipitate.

(c) To another 5 c.c. add a few drops concentrated sulphuric acid, and suspend over the mouth of the test tube a piece of filter paper previously moistened with lead acetate solution.

Discuss the action of the sodium, and the chemistry of the tests (a), (b), (c).

2. Simple Test for "Amid" Nitrogen and for Sulphur.— Put a little protein in a test tube with a few cubic centimeters of strong sodium hydrate solution and three drops lead acetate solution. Heat to boiling, and suspend a piece of litmus paper over the mouth of the test tube. Explain.

3. Test for Phosphorus.—Boil some casein in the hood with 10 c.c. strong nitric acid in an evaporating dish. Evaporate nearly to dryness, add 25 c.c. water, and test for phosphates.

4. Albumins.—Preparation of an albumin solution. Considering "egg white" to contain 12 per cent. of albumin, prepare a 2 per cent. solution by suitable dilution with distilled water. Shake thoroughly, and filter through a plug of cotton.

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* A mixture of casein and dry egg albumen.

5. **Coagulation by Heating.**—Heat a little of the albumin solution in a test tube. Compare the coagulation so obtained with that obtained when the solution is diluted (a) 20 times with distilled water, (b) 20 times with .1 per cent. salt solution, (c) 20 times with .01 per cent. acetic acid, (d) 20 times with equal volumes of .1 per cent. salt solution and .01 per cent. acetic acid solution.

6. **Coagulation Temperature.**—Ascertain the temperature of coagulation of albumin as follows: Faintly acidify a portion of the solution with a few drops of .5 per cent. acetic acid. Filter if necessary. Place a portion of the solution in a test tube, insert a thermometer by means of a cork, and suspend the tube in a large beaker of water. Heat the beaker slowly with a small flame, and observe the point at which the albumin solution becomes cloudy.

7. **Sulphosalicylic Acid Test.**—To some albumin solution in a test tube add a few drops of sulphosalicylic acid solution (25 per cent.). Determine the delicacy of the test.

8. **Nitric Acid Test.**—Put 5 c.c. of the solution in a test tube, and introduce 5 c.c. of concentrated nitric acid very carefully with a pipet to the bottom, forming an under layer. Determine the lowest protein concentration at which the test is unmistakable. Allow 10 minutes if the reaction is slow in appearing.

9. **Picric Acid Test.**—Add to a portion of the albumin solution a few drops of a solution of picric acid (1 per cent.) and citric acid (2 per cent.)—Esbach's reagent. Determine the lowest protein concentration at which this test is unmistakable.

10. **Action of Ammonium Sulphate.**—Add some solid ammonium sulphate to 10 c.c. of the albumin solution in a test tube, shaking frequently until the solution is thoroughly saturated. Allow to stand for a while, occasionally shaking, filter, and test the filtrate for albumin by the heat test. Test the solubility in water of the precipitate on the filter paper,

11. **Action of Magnesium Sulphate.**—Perform a similar experiment, using solid magnesium sulphate instead of ammonium

sulphate. To a portion of the filtrate add one or two drops acetic acid.

12. Biuret Test.—To a portion of the albumin solution add a little sodium hydrate, then, drop by drop, very dilute copper sulphate. The solution becomes violet. Study the delicacy of the reaction. After adding to the albumin solution some solid ammonium sulphate, repeat the test with (a) the same amount of alkali (b) a large amount of 40 per cent. alkali.

13. Millon's Test.—To a portion of the albumin solution add a few drops of Millon's reagent. A precipitate forms, which, on heating, becomes brick red. Repeat, using a dilute solution of phenol instead of albumin. On what group in the protein molecule does this test depend? Add sodium chlorid and repeat the test. Explain.

14. Xanthoproteic Test.—To a few c.c. of the solution add one-third of its volume of concentrated nitric acid; a white precipitate may or may not be produced (according to the concentration and the nature of the protein). Boil. The precipitate or liquid turns yellow. Allow the solution to cool, and add an excess of ammonia. Explain.

15. The Glyoxylic Acid Reaction (Hopkins and Cole).— Treat 2 or 3 c.c. of the solution with the same volume of "reduced oxalic acid." Mix and add an equal volume of concentrated sulphuric acid, pouring down the side of the tube. A purple ring forms at the junction of the fluids. Mix the fluids by shaking the tube gently from side to side. The purple color spreads through the whole fluid. Repeat in the presence of nitrates, chlorates, nitrites, excess of chlorids and carbohydrates, respectively.

"Reduced oxalic acid" is prepared by Benedict's method as follows: To 10 g. powdered magnesium in a flask add a little water, and then add slowly, with shaking and cooling, 250 c.c. of cold saturated oxalic acid solution. Filter, acidify the filtrate with acetic acid, and dilute to one liter.

16. Acetic Acid and Potassic Ferrocyanid.—Acidify some albumin solution in a test tube with acetic acid, and add a few drops

of potassic ferrocyanid solution. A white flocculent precipitate is formed. Determine the delicacy of this precipitation.

17. Alcohol.—Add an excess of alcohol (one or two volumes) to some albumin solution. If the precipitate is small, add a little dilute sodium chlorid solution.

18. Tannic Acid.—Make some protein solution slightly acid with .1 per cent. of acetic acid, and add a few drops of tannic acid solution.

19. Phosphotungstic Acid.—Make a protein solution acid with dilute hydrochloric acid, and add a few drops of the reagent.

Globulins.—The tests are made upon blood serum.

20. Action of Carbon Dioxid.—Dilute 5 c.c. of clear serum with 45 c.c. of ice-cold water. Place the mixture in a cylinder or large test tube, and pass through it a stream of carbon dioxid. What is the effect of too much carbon dioxid?

21. Precipitation by Dialysis.—Pour 20 c.c. of serum into a parchment dialyzing tube previously soaked in distilled water. Suspend the tube, with its contents, in a large volume of water. Explain the precipitation.

Pour serum drop by drop into a large volume of distilled water (in a beaker). What takes place? Explain.

22. Precipitation by Magnesium Sulphate.—Saturate about 5 c.c. of the serum with magnesium sulphate. A heavy precipitate will be formed. Compare this with the action of the same salt on the egg-albumen solution.

23. Precipitation with Ammonium Sulphate.—To 30 c.c. of serum add an equal volume of a saturated solution of ammonium sulphate, thus obtaining a half-saturated solution. Filter off the precipitate, wash two or three times with a half-saturated ammonium sulphate solution, and dissolve in about 60 c.c. of water. This yields a clear solution of paraglobulin. Apply 5 protein tests to this solution.

#**24. Keratin.**—Show that keratin (hair or horn) is a protein.

25. Gelatin.—Make dilute gelatin solution, and with it make 6 tests for protein (including Millon's). Test for sulphur.

26. Phosphoproteins.—Test the solubility of casein in water, dilute acid, dilute alkali, and dilute salt solution.

Make six protein tests on a solution of casein. How would you test for albumin and casein when both are present? Apply to milk and to unknown furnished.

27. Peptones (Proteoses).—For the following experiments use the peptic digestion mixture obtained with the pig stomach (p. 33). Filter, carefully neutralize, heat to boiling (why?), and again filter. Use the filtrate.

To a small portion add 2 or 3 drops of dilute acetic acid and a few c.c. of saturated sodium chlorid solution. Study the effect of heating and cooling on this precipitate.

Apply protein tests described under "albumin," and record the results obtained.

Dialyze about 10 c.c. of the peptone solution against about 100 c.c. of distilled water in a beaker. After 24 hours test the outside water for peptones. Explain.

#**28. Amino-acids, Tyrosin and Leucin.**— For this experiment use the pancreatic digestion mixture prepared for the study of ferment reactions (p. 35).

With a pipet take out 10 c.c. of clear supernatant liquid. Filter this portion if necessary; dilute it with 2 volumes of water, and by means of Mett's tubes determine whether the proteolytic ferment has been destroyed or is still active.

Pour the rest of the digestion mixture without filtering into a good-sized beaker or flask. With continuous stirring or gentle shaking to prevent burning and bumping, heat the digestion mixture until it begins to boil. Some care is needed in this operation because of the presence of alcohol. When the mixture is boiling remove the flame. Note approximately the volume of the mixture, and measure into a test tube some "Merck's dialyzed iron," 8-10 c.c. for each 100 c.c. of digestion mixture. Dump the collodial iron into the digestion mixture, and shake or stir vigorously. Filter.

To the filtrate add a few drops of ammonia and 5-10 g. of bone-black. Boil for a few minutes and again filter. A clear, faintly-colored solution should be obtained.

Pour this last filtrate into an evaporating dish, acidify with a little acetic acid, and boil down to about one-sixth of the original volume.

Transfer the concentrated liquid to a flask or beaker, and set aside in a cool place for a day or two. Tyrosin and leucin crystallize out, the former first and in much greater abundance. Examine the sediment under the microscope.

The isolation of other amino-acids from the mother liquor is much more difficult.

29. Preparation of Cystin (from Wool).—Heat 50 g. of wool in a 500 c.c. flask with 100 c.c. concentrated hydrochloric acid on a water bath until dissolved. A 3-foot glass tube should be inserted to prevent the loss of too much acid liquid. When dissolved boil very gently over a *small* flame for 3-4 hours. Add solid sodic acetate (100-130 g.) until no free mineral acid can be detected in the solution by means of congo red paper. Allow the mixture to stand for 3-5 days. The longer the mixture is allowed to stand, up to 3 weeks, the more cystin is obtained. Filter on a Buchner funnel and wash with cold water. Then dissolve the precipitate in water (150 c.c.) plus 5-10 c.c. concentrated hydrochloric acid, add about 20 g. purified bone-black, and boil 5-10 minutes.

To prepare pure bone-black, let the impure sample stand in an excess of dilute hydrochloric acid over night, filter, and wash with cold water until the filtrate is neutral.

Filter again with suction, heat the filtrate to boiling, and neutralize the hot hydrochloric acid by adding very slowly hot concentrated sodic acetate solution (avoid an excess, test with congo red paper). The precipitate formed consists of cystin, and should be very white and pure. If it is dark colored, re-dissolve in water and a little hydrochloric acid, and repeat the bone-black treatment.

Keep the mixture boiling, and add very slowly the hot sodic acetate solution until the crystallization begins; keep hot, and after a few minutes add cautiously a little more acetate. Well-formed, large, and characteristic crystals should be obtained.

Examine the crystals under the microscope.

Test for sulphur. Test also for tyrosin. (Ordinarily the crystals are quite free from tyrosin.)

Tyrosin can be prepared from the original mother liquor by decolorizing with bone-black and letting it stand in a cold place.

PART VI

URINE ANALYSIS AND METABOLISM

Quantitative urine analysis has no value except in connection with known volumes of urine representing a definite known metabolism period. Even in student exercises involving the learning of methods, only urines which represent a definite metabolism period should be used, and from the analytical figures obtained the total value for the whole urine (and metabolism period) should be calculated.

The standard common metabolism period is twenty-four hours. The only correct way to collect twenty-four hour urines is to begin the metabolism period immediately after passing the night urine in morning. Note the time and then collect all the urine passed up to the same hour the following morning. The reason for this rule is that during the night much of the waste products corresponding to the food intake of the preceding day is passed and in the early morning, before any food has been taken, the excretion is at its lowest level.

Sometimes it is impracticable to collect twenty-four hour urines, and sometimes it is desirable to study the urine representing shorter metabolism periods such as three, four or six hours. Formerly it was not practicable to make use of such short periods, because the analytical procedures required too much urine; the uric acid determination alone required 150 c.c. By the help of the modern colorimetric methods, nearly complete analysis can be made on the basis of three hour urines. For such short metabolism periods it is necessary to drink not less than 200 c.c. of water at the beginning of the period. The period, whether consisting of three hours or twenty-four hours, should begin immediately after passing the night urine.

It is necessary to use some preservative for the urine unless the analysis can be completed within twenty-four hours. Chloroform (2 c.c.) or toluol or xylol should be introduced into the

empty bottle in which the urine is to be collected so as to exclude from the beginning any possibility of bacterial decomposition.

In systematic urine analysis some determinations should be made as soon as possible (within twenty-four hours) while others can be postponed as long as desirable. The uric acid determination should be made the first day, because the uric acid may either fall out as a sediment or may be decomposed as the result of long standing. The creatinin determination should be made within forty-eight hours, because in some urines it is gradually in part converted into creatin. From the standpoint of spontaneous decomposition in well preserved urine the ammonia determination can be postponed indefinitely, but it is usually better not to delay the ammonia determination more than two or three days. In turbid urines there is more or less danger of precipitation of a part of the ammonia as ammonium magnesium phosphate.

The acidity titration should be made within twenty-four hours. Most urines darken very much on standing and the deepened color makes it difficult to see the faint color which marks the correct endpoint of the titration.

Standard figures for the composition of normal twenty-four urines are abundant in the literature. (See *American Journal of Physiology* 13, 45-115, 1905.) Standard figures for three-hour urines are as yet a scarcity. The figures recorded below for the first three-hour morning urines were obtained in 1918 by S. Youngburg. The subjects were medical students.

1. Aëration Method for the Determination of Ammonia.—Measure 25 c.c. of the ammonium sulphate solution previously used for nitrogen determinations (p. 23) into a tall aërometer cylinder. The cylinder is fitted with a two-hole rubber stopper and glass connections so arranged that compressed (outside) air (or laboratory air previously freed from ammonia) is passed to the bottom of the cylinder and out through a calcium chlorid tube filled with dry cotton, and then through a special absorption tube, into a receiver containing water and a known amount of acid. The cotton serves the purpose of holding back traces of solid sodic carbonate, formed by evaporation on the sides of the cylinder.

Add to the ammonium sulphate solution about 10 g. sodium

COMPOSITION OF THREE-HOUR MORNING URINE.

No.	Total N gm.	NH_3–N mg.	Urea–N gm.	Uric Acid mg.	Creatinin mg.	Body wt. K.
1	.87	42	.72	73	176	58
2	1.00	47	.85	66	192	56
3	1.02	68	.82	56	176	59
4	1.02	79	.82	57	224	66
5	1.12	43	.94	48	154	60
6	1.14	74	.97	72	163	64
7	1.18	51	1.00	71	192	66
8	1.23	107	.99	88	204	64
9	1.26	100	1.02	85	240	80
10	1.26	41	1.04	65	222	79
11	1.33	79	1.08	64	220	77
12	1.37	77	1.16	65	190	70
13	1.42	46	1.13	88	203	54
14	1.46	88	1.23	91	183	67
15	1.48	58	1.29	88	229	65
16	1.51	40	1.27	69	190	64
17	1.54	34	1.35	77	212	58
18	1.55	81	1.34	67	186	61
19	1.88	69	1.66	89	247	73
20	2.18	49	1.85	105	236	68

COMPOSITION OF THREE-HOUR MORNING URINE

No.	Volume c.c.	Acidity in c.c. 0.1 N	H_3PO_4 mg.	Total S mg.	Inorganic S mg.	Ethereal S mg.	Cl g.
1	52	34	159	46	30	4	1.64
2	96	6	142	54	31	7	1.08
3	207	30	293	113	39	28	1.11
4	61	38	247	69	51	4	.54
5	204	5	12	51	40	0	.90
6	81	24	86	62	38	2	1.00
7	111	40	317	96	73	6	1.15
8	185	52	381	99	74	7	1.74
9	90	44	374	107	77	5	.62
10	138	6	19	86	50	4	1.15
11	84	35	232	86	60	11	.75
12	66	48	369	114	93	6	.27
13	203	26	241	76	57	4	1.21
14	102	42	283	107	38	8	1.00
15	142	6	193	95	59	22	1.28
16	107	33	142	105	59	24	.76
17	514	13	92	78	56	10	1.64
18	745	3	165	105	81	7	2.62
19	520	17	237	65	47	1	1.57
20	139	10	261	142	93	6	1.29

chlorid, about 2 g. sodic carbonate, and a few drops of kerosene. Do not add any water; the greater the volume the longer it takes to drive off all the ammonia. Pass a very strong air current through the mixture for one and one-half hours, and collect the ammonia, which the air carries off, in a receiver containing 25 c.c. .1 N acid and about 200 c.c. water. Titrate, and compare the result with the figures obtained by distillation (p. 25). If the results are too low, the air current has been too slow, or the aëration process has not been continued long enough to drive off all the ammonia.

In a similar manner determine the ammonia in urine (25 c.c.) and calculate the 24-hour amount. When working with urine it is desirable, though not absolutely necessary, to substitute 10 g. of potassium oxalate for the 10 g. of sodium chlorid. Salts hasten the removal of the ammonia, and oxalate incidentally prevents the (possible) formation of insoluble ammonium magnesium phosphate.

Save the remainder of the ammonium sulphate solution for sulphate determinations.

2. Colorimetric Method for the Determination of Ammonia. — With an Ostwald pipet measure 1 or 2 c.c. of the ammonium sulphate solution into a large Jena test tube (200 x 25 mm.). Choose the amount which contains nearer 1 mg. of nitrogen. Fit the test tube with a two-hole rubber stopper carrying an inlet tube, reaching to the bottom, and an outlet tube. Connect the former with the compressed air jet, and the latter with an absorption tube having small holes drilled through the wall at the end. Insert the absorption tube into a 100 c.c. measuring flask containing 20-30 c.c. of distilled water and 2 c.c. .1 N HCl. Add 2 drops kerosene and a few drops of a solution containing potassium oxalate and potassium carbonate (15 per cent. of each), quickly put the stopper firmly in the tube, and start the air current, gradually increasing its speed for about two minutes. In ten minutes all the ammonia should have been driven over into the receiving flask.

Remove the absorption tube, rinsing it with water, and dilute the contents in the flask to about 75 c.c.

Pipet 10 c.c. of standard ammonium sulphate (p. 103) into another 100 c.c. measuring flask, and dilute with water to 60 c.c.

Nesslerize both solutions according to the directions given in

the alternative colorimetric method (see below) but using only 10 c.c. of Nessler's solution and omitting the addition of sodium hydroxid.

Make the color comparison according to the directions given in the alternative method (see below).

3. Alternative Colorimetric Method for Determination of Ammonia.*—In this method the ammonia is extracted from the urine by gentle shaking with a synthetic aluminate silicate powder sold under the trade name—permutit (by the Permutit Company, New York). Only such preparations as have passed through a 60 mesh sieve and does not pass through an 80-mesh sieve should be used. Powders of any desired degree of fineness are obtainable.

Before applying this method to urine, use it for the determination of ammonia in the ammonium sulphate employed for nitrogen determinations and in the preceding aëration process. Compare the results obtained by permutit with those obtained (a) by distillation (p. 25); (b) by the marco aëration process (p. 95).

The essential mechanical feature of this new reagent for absorbing ammonia is that it is a clean, moderately fine, insoluble powder which gives off very little dust or turbid material to water, and settles, like sea sand, from water in the course of a few seconds. By virtue of this novel feature the (absorbed) ammonia can be separated by decantation from the solution (or urine) which contained it.

The removal of ammonia by this mineral reagent is not an absorptive phenomenon. The reagent is a complex insoluble sodium salt containing active, i. e., easily replaceable, sodium, and the absorption of ammonia involves the replacement of a part of this sodium by ammonia. The chemical affinity of the active group in the reagent for ammonia is remarkably strong so that under suitable conditions the exchange becomes quantitative as far as the ammonia is concerned.

While the chemical reaction involved in the absorption of ammonia by this reagent is apparently a reaction between a solid and a solution, it remains to be said that the solid powder contains about 20 per cent. of water, and if this water of hydration is removed by heat the activity of the reagent is lost. Even gentle dry heat (100° C.) greatly reduces its activity, so that a

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* J. Biol. Chem. XXIX. 329. 1917.

freshly purified and rapidly dried product is less active than the same product allowed to dry at ordinary temperatures, or than the same product dried rapidly at 100° C., and allowed to "weather" for a day or two.

An important characteristic of this reagent for the absorption of ammonia is that it does not appreciably deteriorate by being used. After washing away the Nesslerized ammonia and surplus alkali first with water, then with one portion of 2 per cent. acetic acid, then once more with water, the powder remaining is just as efficient as before for the absorption of more ammonia.

The process for the colorimetric determination of ammonia in urine by the help of the synthetic zeolite powder is as follows:

Transfer about 2 gm. of the powder to a 200 c.c. volumetric flask. Add about 5 c.c. of water (no more), and with an Ostwald pipette introduce 1 or 2 c.c. of urine, or with a 5 c.c. pipette introduce 5 c.c. of previously diluted urine (corresponding to 1 or 2 c.c. of the original urine). With urines extraordinarily poor in ammonia it may be necessary to use more urine (5 c.c.), but, in so far as it is practicable, it is better not to use more than 2 c.c. and to employ a weaker standard (0.5 mg. of ammonia nitrogen) for the color comparison. Our reason for not wishing to use more than 2 c.c. of urine is based partly on practical experience and partly on the recognition of the fact that the salts in the urine tend to prevent the ammonia absorption from being quantitative. Rinse down the added urine by means of a little water (1 to 5 c.c.), and shake gently but continuously for 5 minutes. Rinse the powder to the bottom of the flask by the addition of water (25 to 40 c.c.) and decant. Add water once more and decant. (In the case of urines rich in bile it is advisable to wash once or twice more). Add a little water to the powder, introduce 2 c.c. of 10 per cent. sodium hydroxid, shake for a few moments and set aside, while preparing the standard ammonium sulphate solution as follows:

Transfer 10 c.c. of the standard ammonium sulphate solution (p. 103) containing 1 mg. of nitrogen to another 200 c.c. volumetric flask and add 2 c.c. of 10 per cent. sodic hydrate (to balance the alkali added to the permutit mixture in the other flask). Dilute to about 150 c.c. and mix. Transfer 20 c.c. of Nessler's solution (see p. 203) to a measuring cylinder. Now give the volumetric flask a vigorous whirl so as to set the solution spinning within the flask and add at once the whole of the Nessler

solution in the cylinder. With another whirling movement secure the complete mixing of the contents in the flask. If the process of Nesslerization has been successful a deep red but *crystal clear* solution is obtained. If it is not perfectly clear throw it away and prepare a fresh standard. With a little experience no trouble is encountered in getting clear solutions. When the standard solution is thus satisfactorily Nesslerized, dilute the contents in the flask containing the permutit and the urinary ammonia to about 150 c.c., whirl the mixture and add the Nessler reagent (20 c.c.) exactly as in the case of the standard solution. Dilute the contents of both flasks to volume (200 c.c.) and make a quantitative color comparison by means of the colorimeter.

Those who are inexperienced in colorimetric work should not fail to observe the following precautions. Do not spill Nesslerized or other alkaline solutions on the mirror of the colorimeter; a single such spill unless immediately washed off with an abundance of cold water ruins the mirror. The most frequent cause of spilling is the placing of too much solution in the colorimeter cups. These cups should be only a little more than half filled. Always rinse the colorimeter cups and the plungers with cold water after using, as a red sediment is otherwise gradually deposited on both. Such sediment, when formed, can be removed by means of a dilute solution of potassium iodide.

Before attempting to determine the color of the unknown the correctness of the instrument, and of the eye, must be ascertained. Rinse both colorimeter cups with the standard Nesslerized solution and fill both with the same solution a little more than half full. Put them in place and set both colorimeter plungers at a height of exactly 20 mm. Adjust the focus of the instrument so that the line dividing the two fields is clear and distinct. By the help of the mirror and by turning the whole instrument, adjust it to the source of light until the two fields look alike. Do not stare at the field too long; close the eye frequently so as to avoid fatigue. If the fields can not be made to look alike the zero point or the optics of the instrument must be wrong and suitable allowance must be made for this error. Having learned to see the fields alike, change the height of one of the plungers and then make a color comparison of the standard against itself readjusting the moved plunger until the fields again look alike. The error should not exceed 0.2 mm. Now set the two plungers again at 20 mm. and ascertain once more that they look alike. Then empty one cylinder, rinse it, and also the plunger, with the unknown; half fill the cup with the un-

known, and make the final color comparison fairly rapidly, before the memory of what the fields looked like when they were equal has been blurred.

The ammonia content is inversely proportional to the depth of the color, provided that one is not more than one and one-half times as deep as the other. Twenty, the depth of the standard in mm., divided by the reading of the unknown, in mm., gives the ammonia nitrogen, in mg., in the volume of urine (or ammonium sulphate solution) taken for the analysis.

Calculate the 24-hour quantity of the ammonia as NH_3 and as ammonia-N, and compare with the figures given by the macro aëration method.

4. Clinical Method for the Determination of Ammonia.—The special reagents required in this determination are (a) saturated potassium oxalate solution and (b) formalin; both of which must be neutral to phenolphthalein. To each reagent add a little of the indicator and .1 N alkali to a faint pink coloration.

To 25 c.c. of urine add about 5 c.c. of the neutralized oxalate solution and 2-3 drops phenolphthalein solution. Titrate the acidity of the urine to a *faint* but unmistakable end point. Then add about 5 c.c. of the neutralized formalin and again titrate to the same degree of coloration as in the preceding titration. Each c.c. of the .1 N alkali used in this titration corresponds to 1 c.c. .1 N ammonia.

The formaldehyd combines with the ammonia giving neutral hexamethylenetetramin, thus setting free acid equivalent to the amount of ammonia present.

5. Total Nitrogen. Kjeld. **Colorimetric Method.**—Special equipment called for: (a) Duboscq colorimeter, (b) standardized 1 c.c. "Ostwald pipets," with extra long stems, (c) a solution of specially purified ammonium sulphate, of such strength that 10 c.c. contain 1 mg. of nitrogen (0.4716 g. salt per liter, (d) modified Nessler-Winkler reagent (*see* p. 203).

This determination requires 0.7 to 1.5 mg. of nitrogen. The total nitrogen in urine is on the average about 25 times as much as the "ammonia" nitrogen. Dilute 5, 10 or 20 c.c. of urine to 100 c.c., mix and with an Ostwald pipet transfer 1 c.c. of the diluted urine to a large hard glass test tube. (This pipet must

be drained for 15 seconds against the wall of test tube and then blown clean.) With an ordinary pipet add 1 c.c. of the phosphoric-sulphuric acid-copper sulphate mixture together with a pebble, to prevent bumping. Heat over a micro burner (no hood necessary) until the water is driven off and fumes become abundant within the tube. This should take place in about two minutes. When filled with fumes close the mouth of the test tube with a watch glass and continue the boiling at such a rate that the tube remains filled with fumes yet almost none escape. Within two minutes after the mouth of test tube was closed the contents should become clear, and bluish or light green. Continue the gentle boiling for 30 to 60 seconds longer, provided, however, that the total boiling period, with test tube closed, must not be less than two minutes. Remove the flame and let cool for a little less than two minutes, then add water. Rinse the hot digestion mixture (sometimes turbid from silica) into a 200 c.c. volumetric flask, using for this purpose about 125 c.c. of water.

Transfer 10 c.c. of standard ammonium sulphate solution containing 1 mg. of nitrogen into another 200 c.c. volumetric flask. Add 1 c.c. of the concentrated phosphoric-sulphuric acid mixture, to balance the acid in the unknown, and dilute to a volume of about 150 c.c. When both flasks are thus ready give each flask a whirl and add 30 c.c. of Nessler's reagent. Shake a little more and dilute both flasks to the 200 c.c. mark.

If the unknown Nesslerized digestion mixture is turbid, centrifuge a portion, giving a crystal clear fluid above a white sediment (silica). If the sediment is colored the Nesslerization was not successful and the determination must be discarded. Determine the color value of the centrifuged solution as described under the permutit method for determining ammonia and calculate the total nitrogen in the three-hour or twenty-four hour quantity of urine.

Determine the nitrogen in 5 c.c. of the undiluted urine by the macro Kjeldahl process and compare with the value given by the colorimetric process. The two methods should give substantially identical values.

6. **Reactions of Urea.**—Put a crystal of urea on a glass slide or a watch-glass and cover it with a drop of water. With a glass rod, put a drop of nitric acid next to this. Let the two drops run together, and notice the precipitation of urea nitrate at the junction. Examine under the microscope, and sketch the crystals.

Put a few crystals of urea into a dry test tube, and heat till they melt. With moist litmus paper test the reaction of the fumes given off. Explain.

Cool the test tube. To the residue, consisting of biuret and cyanuric acid, add a little water, filter, and with the filtrate make the biuret test.

Dissolve a few crystals of urea in 5 c.c. water in a test tube. Test its reaction with litmus paper. Heat the solution to boiling and test the steam with moist litmus paper. Cool the liquid and test with litmus paper. Explain.

To a solution of urea in a test tube add an equal volume of sodium hypobromite solution. Make this by mixing and cooling equal volumes of bromin solution and 40 per cent. sodic hydrate solution.

The reaction with sodium hypobromite has been used for the quantitative determination of urea, but as ordinarily used for this purpose the method has very little value.

7. Colorimetric Method for Determination of Urea (*J. Biol. Chem.* 26, 501, 1916; 38, 111, 1919).—Merck's blood charcoal was a necessary reagent in the determination of urea in urine by the direct Nesslerization process of Folin and Denis. By using urease preparations sufficiently free from nitrogenous materials the urea nitrogen can be Nesslerized without any charcoal treatment.

Urease Preparation.—Wash about 3 g. of permutit in a flask once with 2 per cent. acetic acid, then twice with water; add 5 g. of fine Jack bean meal and 100 c.c. of 15 per cent. alcohol (16 c.c. of ordinary alcohol plus 84 c.c. of water). Shake gently but continuously for 10 to 15 minutes, pour on a large filter and cover with a watch glass. The filtrate contains practically the whole of the urease and extremely little of other materials. The urease solution will keep for about a week at room temperatures and for 4 to 6 weeks in an ice box.

Buffer Mixtures for Urease Decompositions.—Mixtures of mono- and disodium phosphates in the proportion 1 molecule of the former to 2 of the latter, and in molar concentrations, are usually employed to preserve a substantially neutral reaction during the decomposition of urea by means of urease. Dissolve 69 g. of monosodium phosphate and 179 g. of crystallized di-

sodium phosphate in 800 c.c. of warm distilled water. Cool and dilute to a volume of 1 liter.

It would seem to be rather doubtful whether the maintenance of neutrality is adequate to fully explain the accelerating action of phosphates on the urea decomposition, because pyro- and metaphosphates seem to be more effective than orthophosphates. An excellent buffer mixture is obtained by dissolving 14 g. of sodium pyrophosphate ($Na_4P_2O_7$, $10H_2O$) in enough half normal phosphoric acid to make a volume of 100 c.c. The half normal phosphoric acid is made by diluting 20 c.c. of 85 per cent. phosphoric acid to 1 liter and titrating 5 c.c. with tenth normal alkali and phenolphthalein as indicator to a faint pink color. On the basis of this titration dilute the acid to a substantially correct half normal solution. Metaphosphoric acid is fully as good as phosphoric acid, but it is much more difficult to prepare a solution of the requisite degree of neutrality with metaphosphoric acid because of its variable water and free phosphoric acid content. The pyrophosphate-phosphoric acid mixture, the preparation of which is described above, gives a faint color with rosalic acid. 5 c.c. when titrated with tenth normal alkali and phenolphthalein should give a faint but distinct color with about 18 c.c. of the alkali.

Transfer with an Ostwald pipet 1 c.c. of diluted urea solution or urine (dilution sometimes 5 or 20, but usually 10 c.c. diluted to 100 c.c.) to a clean test tube; add 1 or at the most 2 drops of buffer mixture and 1 c.c. of urease solution. Digest in a beaker of warm tap water (40° to 55° C.) for 5 minutes or at room temperatures for 15 minutes. At the end of the digestion period rinse the contents of the test tube into a 200 c.c. volumetric flask and dilute to a volume of about 150 c.c.

Transfer 1 mg. of N in the form of ammonium sulphate to another 200 c.c. volumetric flask; to this standard add 1 c.c. of urease solution and dilute to about 150 c.c. Then add with shaking (with a cylinder) 20 c.c. of Nessler solution to each. Dilute to volume and make the color comparison, never omitting to first read the standard against itself.

The height of the standard (usually 20 m.m.) divided by the height of the unknown, gives the nitrogen, in mg., present in the fraction of a c.c. of undiluted urine present in the 1 c.c. of diluted urine taken for the analysis.

Unless the colorimetric reading is between 14 mm. and 30 mm.

the determination should be repeated with 1 c.c. of urine so diluted as to give readings coming within those limits. Calculate the total urea-N and subtract the preformed ammonia-N.

A few explanatory remarks may be added. Many kinds of biological nitrogenous materials, particularly amino acids, peptones and albumins, prevent the development of the color reaction given by ammonia and Nessler's reagent. This was first discovered in attempts to determine by direct Nesslerization the ammonia formed in pancreatic digestion mixtures. If very little such nitrogenous material is present the result obtained is deceptive for then the color is merely diminished and the error will not be detected. The careful observer will find, however, that in such cases the color obtained tends to be visibly more greenish and less distinctly red than the standard. Because of the serious interference caused by albuminous materials it may be thought that the procedure described above is not applicable to albuminous urines, but a series of determinations have shown that even urines very rich in albumin have in fact so little in comparison with the amount of urea present that correct results are invariably obtained by direct Nesslerization.

Because of the extremely low nitrogen content of our urease preparation it is not really essential that the urease should also be added to the standard ammonia solution, but we have thought it best to recommend that it be added simply as a precaution against the possible occurrence of less good urease preparations. In recommending the addition of the urease to the standard as well as to the urine we have also had in mind the probability that some will omit the use of the permutit when making the alcoholic urease extracts and will then have variable small traces of ammonia in their extracts.

The reason why the urease decomposition had best be made in test tubes rather than in volumetric flasks is to avoid failure due to the use of flasks which have been used for Nesslerization purposes. Such flasks may look perfectly clean, but, unless they have been rinsed with nitric acid, they will contain enough mercury compounds to destroy entirely the urease and scarcely a trace of ammonia is obtained.

Compare the urea-N value of the urea solution with that obtained by the Macro Kjeldahl process.

#8. **Uric Acid Preparation of Uric Acid from Urine.** — To 500 c.c. of urine add 25 g. of ammonium sulphate, stir until the sulphate is dissolved, and add about 10 c.c. of ammonia. Let stand over night, filter, and wash two or three times with water containing a little ammonia. Transfer the precipitate to a small

beaker, add a few drops of hydrochloric acid, and let stand till the following day. Examine the crystals under the microscope.

9. Murexid Test for Uric Acid.—Place a few uric acid crystals on a porcelain crucible cover. Add three drops of strong nitric acid. Heat cautiously, blowing on the liquid to complete dryness. A red color should appear. Let cool and add a few drops of dilute ammonia. Repeat with caffein. Explain.

10. Phosphotungstic Acid Test for Uric Acid.—Dissolve a few crystals of uric acid in 2 c.c. very dilute sodic hydrate solution. Add 1 c.c. of "qualitative uric acid reagent" (*see* p. 207). Then add 10 c.c. of saturated sodium carbonate solution. A pronounced blue coloration should be obtained. Repeat the reaction with 5 c.c. of urine.

11. Colorimetric Method for the Determination of Uric Acid. (*Journal of Biol. Chem.* 38, 1919).— Transfer 1-3 c.c. of urine, according to concentration, to a centrifuge tube and add water to a volume of about 6 c.c. Add 5 c.c. of a silver lactate solution (silver lactate 5 per cent., lactic acid 5 per cent.), and stir with a fine glass rod. Rinse off the rod with a few drops of water. Centrifuge the counterbalanced tube for 2-3 minutes. Add a drop of silver lactate solution so as to be sure that an excess is present; if a precipitate (of AgCl) is formed, add 2 c.c. more of the silver solution and centrifuge again; if no precipitate forms, pour off the liquid as completely as possible.

To the precipitate in the centrifuge tube add, from a buret, 4 c.c. of a 5 per cent. sodium cyanid solution (poisonous—3 c.c. may be fatal dose). Stir the mixture until a perfectly clear solution is formed. Rinse the stirring rod, collecting the rinsings in a 100 c.c. graduated flask; pour the contents of the tube into the same flask and rinse the tube 3 times with about 5 c.c. of water. Add 5 c.c. of a 10 per cent. sodium sulphite solution (to balance the sulphite in the standard uric acid solution) and dilute to a volume of about 40 c.c. In another 100 c.c. flask place 5 c.c. of a standard uric acid-sulphite solution (see below) containing 0.5 mg. of uric acid; add 4 c.c. of cyanid solution and about 35 c.c. of water. Then add 20 c.c. of 20 per cent. sodium carbonate solution to each flask and finally add *with shaking* 2 c.c. of the uric acid reagent described on p. 207. Let stand 3-5 minutes, fill to the mark and mix.

Set the standard uric acid solution at 20 m.m. in both colorimeter cups and adjust the colorimeter until the two fields are exactly alike. Then determine the color of the unknown. Since the standard is only 0.5 mg., 10 divided by the reading of the unknown (in mm.) gives the amount of uric acid (in mg.) in the volume of urine taken.

Caution.—Be careful to pour the discarded blue uric acid cyanid mixtures directly into the drain pipes of sinks. If sinks contain acids a gaseous mixture of CO_2 *and HNC will be set free.*

The uric acid reagent gives an intense blue color with uric acid in the presence of an alkali. The same blue color is obtained with some other substances, notably with some phenol derivatives present in urine. It is therefore necessary to separate the uric acid from these products before applying the color reaction. Acidified silver lactate is used for the precipitation of the uric acid. In the presence of sodium chlorid it carries down every trace of uric acid, provided that an excess of the silver salt is added. The sodium cyanid dissolves the silver precipitate and sets free the uric acid; it also greatly reduces the tendency of the color to fade on standing. The use of the cyanid first introduced by S. R. Benedict has greatly simplified the uric acid determination.

Preparation of Standard Uric Acid Solution.—In a 500 c.c. flask dissolve exactly 1 g. of uric acid in 150 c.c. of water by the help of 0.5 g. lithium carbonate. Dilute to 500 c.c. and mix. Transfer 50 c.c. to a liter flask; add 500 c.c. of 20 per cent. sodium sulphite solution; dilute to volume and mix. Transfer to small bottles (cap. 200 c.c.) and stopper tightly. This standard uric acid solution keeps almost indefinitely in unopened bottles, because the sulphite prevents the spontaneous oxidation of the uric acid. In used bottles the standard usually remains good for 2-3 months.

12. Creatinin.—Transfer to a small bottle about 3 g. dry picric acid. Add about 100 c.c. of urine, insert a cork, and shake continuously for ten minutes. Filter a portion of the mixture and transfer 5 c.c. of the filtrate to a bottle or flask (capacity not less than 250 c.c.).

Measure 5 c.c. of the original urine into another similar flask or bottle. Add to each, first 20 c.c. saturated picric acid solu-

tion, and then 5 c.c. 10 per cent. sodic hydrate. Let stand 5-10 minutes and add 200 c.c. tap water to each.

Remove by decantation the liquid from the bottle containing urine and picric acid, and to the sediment add about 25 c.c. water and 15 c.c. sodic hydrate (10 per cent.). Shake, let stand for a few minutes, and fill the bottle with tap water.

The substance in urine which is precipitated by picric acid and which gives a deep red color with alkaline picrate solutions, is creatinin. No other known substance occurring in normal urine gives this color reaction. Therefore, on the basis of this reaction, it is easy to determine the creatinin quantitatively.

13. Quantitative Determination of Creatinin.—A suitable and convenient "creatinin reagent" is prepared by adding 75 c.c. of 10 per cent. sodic hydrate to a liter of saturated picric acid solution. If the picric acid is pure and the alkaline solution is kept away from the light and from dust it keeps well for several days. It is usually more safe, however, to prepare only so much of the solution as is used up the same day. For a single determination it is not worth while to prepare the reagent; employ instead the picric acid solution and the alkali, using 20 c.c. of the former and 15 c.c. of the latter.

By means of an Ostwald pipet transfer 1 c.c. or 2 c.c. urine to a 100 volumetric flask. To another similar flask transfer 1 c.c. of a standard creatinin solution (1.61 g. of creatinin zinc chlorid dissolved in one liter of tenth normal hydrochloric acid), 1 c.c. of which contains 1 mg. of creatinin. To each flask add 20 c.c. of picric acid solution, then add from a buret 1.5 c.c. of 10 per cent. sodic hydrate to each, and let stand for ten minutes. If the alkaline picrate solution is used, add, with a cylinder, 20 c.c. to each flask. At the end of ten minutes dilute to the mark with water and mix.

Read the standard against itself in the colorimeter at 20 mm. until the correct value (20 mm.) can be obtained. The error in reading should not exceed .2 mm. Rinse the right-hand cup and prism with the unknown, and determine its color in terms of the standard set at 20 mm. Twenty divided by the reading gives the creatinin in milligrams in the quantity of urine taken (1-5 c.c.).

Calculate the total creatinin.

#**14. Creatin.**—Unless considerable meat or fish has been eaten the urine of normal adults contains only traces of creatin.

Urines of children and of sick persons, particularly fever patients, appear, on the other hand, to contain relatively considerable quantities of creatin (.2 g. to 1 g. or more per day in fever patients).

Creatin is determined in such urines by first converting it into creatinin. This is done as follows:

Measure the urine (usually 1 c.c.) into a flask (capacity 300 c.c.) and add 20 c.c. saturated picric acid solution (not the creatinin reagent). Weigh flask and contents and add about 150 c.c. water. Boil gently for 45 minutes, then more rapidly until the original volume (determined by weighing) is obtained (a variation of 3 or 4 g. makes no appreciable difference). Cool. Add 1.5 c.c. 10 per cent. sodic hydrate, let stand 10 minutes, and compare, as in the case of preformed creatinin, with the color obtained from 1 mg. creatinin.

Twenty divided by the reading gives the sum of the creatin and creatinin present.

Calculate the total quantity and subtract the preformed creatinin.

If an autoclave is available, the conversion into creatin can be made more rapidly.

Measure the urine (1 c.c.) into a 100 c.c. volumetric flask, and add 20 c.c. saturated picric acid solution. Cover the mouth of the flask with tinfoil, and heat in the autoclave at 115°-120° for 20 minutes. Cool, add 1.5 c.c. sodic hydrate, and finish the determination in the usual manner.

15. Hippuric Acid.—Take with the evening meal 2 g. of sodium benzoate, and collect the urine until next morning.

Evaporate to small volume and transfer with a little wash water to a small flask. Acidify strongly with sulphuric acid and put away for twenty-four hours. Filter and dry the precipitate, consisting of hippuric acid, uric acid, and other substances. Extract the hippuric acid with acetic ether. Set aside for spontaneous evaporation. Examine microscopically. Heat the dry substance in a dry tube, and note the odor of bitter almonds (benzaldehyd).

16. Determination of Inorganic Sulphates.— (*J. Biol. Chem.*, 1, 131).—Transfer to a 250 c.c. beaker 25 c.c. of the ammonium sulphate solution in which the ammonia was determined (p. 23).

Dilute with water to about 100 c.c.; add 10 c.c. of 20 per cent. sodium chlorid solution and 5 c.c. of concentrated hydrochloric acid.

The addition of sodium chlorid is necessary only when the sulphate determination is to be made in ammonium or potassium sulphate solutions. Such solutions give too low sulphate values unless sodium chlorid is added. The reason is rather obscure, but in general it may be stated that the sulphate precipitate obtained is practically never pure $BaSO4$, but by adding sodium chlorid the precipitate obtained has been found, empirically, to give the weight corresponding to $BaSO4$. Urine contains so much more of sodium than of ammonium and potassium salts that in urine analysis the addition of sodium chlorid is not required.

Fill a buret with 5 per cent. barium chlorid solution and place the beaker, containing the sulphate solution, under it so that the buret will deliver on the side (the spout) of the beaker. Add thus, drop by drop, 10 to 15 c.c. of barium chlorid. The contents in the beaker must not be stirred or agitated to any degree while the barium is added. If $BaSO_4$ is formed too rapidly in the cold much "occlusion" takes place, and, in addition, the precipitate is apt to be so fine that it will pass through when filtering. After the beaker has stood for 10 to 30 minutes mixing the contents of the beaker will do no harm and must be done to complete the precipitation. Let stand for 1 to 24 hours, according to convenience, before filtering. (The ethereal sulphates are not hydrolyzed in the cold.)

Prepare an asbestos mat as follows: Fill the Gooch crucible with the freshly shaken asbestos suspension once, or at the most twice. Pack the asbestos down by vigorous suction (water pump). The mat should be about 1 mm. thick. Then cover the mat with a perforated porcelain plate or with a layer of sea sand, 5 to 7 mm. thick. The sand should previously be kept for at least 24 hours in contact with 5 per cent. hydrochloric acid. Wash the asbestos and covering with water and moderate suction until the filtrate is free from asbestos particles and water clear. Make as dry as possible by suction. Heat very gently at first (not over 100° C.) to drive off the steam without mechanical disturbance of the mat; then ignite; cool for 15 to 20 minutes, and weigh. The same mat can be used for several successive sulphate determinations provided that adequate care is taken not

to disturb the mat, either by too rapid use of water when filtering or by steam during the heating.

Transfer the barium sulphate to the Gooch crucible and wash 6 to 10 times with distilled water. Heat to dryness, ignite, cool and weigh. From the weight of the $BaSO_4$ obtained, calculate the ammonium sulphate and compare with the weight known to be present. Repeat with 25 c.c. of urine to which has been added about 75 c.c. of water and 5 c.c. of hydrochloric acid. (No sodium chlorid need be added when working with urine.) Calculate the results as S and as H_2SO_4.

17. Determination of Total Sulphates.—In this determination the ethereal sulphates must be hydrolyzed by hydrochloric acid and heat before barium chlorid is added. Transfer 25 c.c. of urine to a beaker, add about 25 c.c. of water and 5 c.c. of concentrated hydrochloric acid. Cover with a watch glass and boil gently for 20 to 30 minutes. Then dilute to about 100 c.c., heat to boiling and with a pipet add 10 c.c. of 5 per cent. barium chlorid solution. Let stand for an hour, or as much longer as may be convenient. Filter, wash, ignite, cool and weigh, as in the case of the inorganic sulphate determination.

Calculate as S and as H_2SO_4 and, by subtraction of the corresponding values obtained as inorganic sulphates, calculate the S and H_2SO_4 present in the form of ethereal sulphates.

18. Determination of Total Sulphur.— (Benedict, *J. Biol. Chem.*, 6, 363; Denis, *J. Biol. Chem.*, 8, 401.)—Transfer 25 c.c. of urine to a porcelain dish (diam. 10-12 cm.); add 5 c.c. of a solution containing 25 per cent. of copper nitrate and 25 per cent. of sodium chlorid, and 10 per cent. of ammonium nitrate. Evaporate to dryness on the water bath. Then heat over a flame, preferably over a Fletcher burner; the burners attached to the copper condensers used in Kjeldahl determinations are also good. The heat should be very moderate at first and should then be gradually increased until the dish becomes almost red hot, continue the vigorous heating for 10 minutes so as to decompose and drive off all of the nitric acid fumes. The organic matter, including the sulphur compounds, are thus oxidized, but the sulphates formed do not escape; they are held back as copper sulphate. The sodium chlorid present in the oxidizing nitrate mixture serves to prevent mechanical losses due to the explosive

violence with which the oxidations are apt to occur without the presence of the sodium chlorid. Allow the dish to cool. Add 20 c.c. of 10 per cent. hydrochloric acid and warm quickly. Filter the dissolved contents into a beaker, using for this purpose 75 to 100 c.c. of hot water. Heat to boiling and add slowly 15 c.c. of 5 per cent. barium chlorid solution. Let stand an hour or longer, filter on the asbestos mat, wash, ignite, cool and weigh.

Calculate as S and as H_2SO_4 and by subtraction of the S and H_2SO_4 found as total sulphates, calculate the values corresponding to the "neutral" or "unoxidized" sulphur.

19. Phosphates.—Add a few drops neutral calcium chlorid solution:

(a) to 5 c.c. 1 per cent. monopotassium phosphate solution,
(b) to 5 c.c. 1 per cent. disodium phosphate solution,
(c) to a mixture of the two phosphate solutions,
(d) to 5 c.c. turbid urine,
(e) to 5 c.c. clear urine,
(f) to 5 c.c. of urine after filtering off the precipitate obtained by the addition of a little ammonia. Explain the results.

20. Determination of Phosphates.—STANDARD PHOSPHATE SOLUTION.—Dissolve 4.39 g. pure monopotassium phosphate in water and dilute to 500 c.c. Each c.c. contains 2 mg. phosphorus. Label and preserve.

STANDARD URANIUM SOLUTION.—Dissolve 18 g. uranium acetate and 50 c.c. 50 per cent. acetic acid in water. Dilute to 500 c.c. If turbid, allow to settle (for a day or two), and remove the clear supernatant solution by means of a siphon.

Transfer 25 c.c. of the phosphate solution to a flask, add 5 c.c. special sodic acetate solution (containing 10 per cent. acetate and 3 per cent. acetic acid), heat to boiling, and add 15-20 c.c. of the clear uranium solution from a buret. Heat again to boiling, and now add the uranium slowly until 2 drops of the phosphate uranium mixture when added (by means of a glass tube drawn out to a point like a pipet) to a minute pinch of powdered potassium ferrocyanid (on a white plate) gives a faint yet unmistakable brownish coloration.

Repeat until the exact titrating value of the uranium solution has been ascertained.

Calculate the value of the uranium solution in terms of phosphorus (P) and also as H_3PO_4. Label and preserve.

DETERMINATION OF PHOSPHATES IN URINE.—Measure 50 c.c. of urine into a flask, add sodic acetate, heat to boiling, and titrate with the uranium solution, exactly as in the case of the standard phosphate solution. After having found the approximate phosphate content by means of the preliminary titration, repeat, adding nearly all the required uranium solution at once to the hot urine, and finish by adding only a few drops at a time.

Calculate in terms of phosphorus and of phosphoric acid the phosphate content of the 24 hour urine under examination.

21. Acidity of Urine.—(See *Am. Journ. of Physiology*, 9, 265, 1903, and 13, 102, 1905.)

Nearly all of the titratable acidity of urine is due to acid phosphate. The end point of this titration is not very sharp, partly on account of the color of the urine (compare p 91) and partly because of the presence of ammonium salts. The titration is further complicated by the presence of calcium, for when alkali is added to a mixture of phosphate and calcium some tribasic phosphate is precipitated. In the presence of sodium or potassium oxalate the premature formation of tribasic salt is prevented. But, on account of the ammonium salts, there is still danger of over-titrating and the first distinguishable coloration should be taken as the end point. With phenolphthalein as indicator titrate the acidity of the monopotassium phosphate (20 c.c.) used for standardizing the uranium solution.

Repeat after having added 5 c.c. of neutral calcium chlorid solution (2 per cent).

Repeat after having added 5 c.c. of the calcium chlorid solution and 5 c.c. of saturated neutralized potassium oxalate solution.

Note.—For accurate work 15 g. of solid neutral potassium oxalate should be used instead of the oxalate solution prescribed, but it is rather difficult to obtain strictly neutral oxalate.

Transfer 20 c.c. of undiluted urine to a small flask or beaker, add 5 c.c. of the oxalate solution. With another sample of the same urine in another flask or beaker as a guide, and with phenolphthalein as indicator, titrate until the oxalated urine becomes a shade darker than the other.

Calculate the acidity for the whole urine in terms of tenth normal acid.

Calculate the phosphate of the same urine in terms of tenth normal phosphoric acid, but considering the H_3PO_4 as a monobasic acid. Compare the two values and calculate what per cent. of the phosphate is present as acid phosphate. The remainder, if any, is dibasic phosphate.

Compare also the acidity and the acid phosphate with the ammonia, expressed in c.c. of tenth normal solution. The ammonia usually varies more or less directly with the acid phosphate (and the acidity).

✱ **22. Determination of Chlorids.**—STANDARD SILVER NITRATE SOLUTION.—This is prepared by dissolving 23.94 g. silver nitrate per liter of solution (or 5.99 g. in 250 c.c.).

STANDARD AMMONIUM SULPHOCYANATE SOLUTION.—Dissolve 6 g. of the salt in 800-900 c.c. water. Transfer 10 c.c. of the silver solution to a beaker or flask; add 50 c.c. water, 5 c.c. concentrated nitric acid, and 2 c.c. of saturated ferric ammonium sulphate solution. By means of a buret titrate the acidified silver solution with the sulphocyanate solution. On the basis of the result, dilute a part of the sulphocyanate solution so as to give 500 c.c. (or a liter) of a solution, 20 c.c. of which is exactly equivalent to 10 c.c. of the silver solution.

Each c.c. of the silver solution corresponds to 5 mg. chlorin (or to 8.23 mg. sodium chlorid).

The chlorin determination in urine is carried out as follows:

Pipet 10 c.c. of urine into a 100 c.c. volumetric flask, add 50 c.c. distilled water, 5 c.c. saturated ferric alum solution, and 5 c.c. concentrated nitric acid. Add 20 c.c. standard silver nitrate solution, fill up to the mark with distilled water, and shake. Filter through a dry filter into a dry beaker or flask. With a clean, dry pipet transfer 50 c.c. of the filtrate to another beaker, flask, or evaporating dish, and titrate in the same manner as when standardizing the silver solution.

Since the sulphocyanate solution is half as strong as the silver solution, and since only one-half of the surplus silver was taken for titration, 20 minus the sulphocyanate titration figure represents the silver nitrate which has combined with the chlorin of the urine to form silver chlorid. This figure multiplied by 5 or by 8.23 gives the chlorin or sodium chlorid (in milligrams) present in 10 c.c. of urine.

Calculate the twenty-four hour quantity.

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23. Simplified Chlorid Determination.—The removal of the silver chlorid precipitate in the preceding method is generally conceded to be necessary, because of the fact that a part of the silver chlorid is converted into silver sulphocyanate during the titration, unless thus removed. The error due to this side reaction when the chlorid is not removed is a loss of about 0.05 c.c. of sulphocyanate for 5 c.c. of urine, a loss amounting to a deficit of about 0.1 g. of sodium chlorid per liter of urine.

Standard silver nitrate. Dissolve 7.28 g. of silver nitrate and dilute to a volume of 250 c.c. 1 c.c. is equivalent to 10 mg. of $NaCl$.

INDICATOR.—To 100 g. of ferric ammonium sulphate add 100 c.c. of water and 200 c.c. of concentrated nitric acid. 5 c.c. of the resulting solution is taken for each titration.

STANDARD AMMONIUM SULPHOCYANATE.—Dissolve 2 g. of ammonium sulphocyanate in 200 c.c. of water and mix. Transfer 10 c.c. of the standard silver nitrate solution to a beaker, add 20 c.c. of distilled water and 5 c.c. of the indicator. Fill a buret with the sulphocyanate solution and titrate with constant stirring until the characteristic reddish end point is reached. On the basis of the figure obtained prepare 200 or 250 c.c. of ammonium sulphocyanate solution, which is equivalent to the standard silver nitrate solution. With a pipet transfer 5 c.c. of urine to a beaker, add 20 c.c. of water, 5 c.c. of indicator and finally (with a pipet) 10 c.c. of silver nitrate solution. While stirring with a glass rod, titrate the surplus silver with the standard sulphocyanate solution until the first faint but unmistakable brown or reddish coloration is obtained. On standing or continued stirring the color would disappear, so the very first end point must be taken.

Subtract the ammonium sulphocyanate used (in c.c.) from 10 and multiply by 10. This gives, in mg., the amount of sodium chlorid present in 5 c.c. of urine. Calculate the twenty-four hour quantity.

24. Indican.—To 10 c.c. of urine add 2 c.c. of copper sulphate solution, 5 c.c. chloroform, and an equal volume (12 c.c.) of strong hydrochloric acid. Close the mouth of the tube with the thumb, and cautiously invert a few times.

The amount of indican present is proportional to the depth of color of the chloroform extract. This qualitative test is often

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made roughly quantitative by using the color of Fehling's solution as a standard.

25. Metabolism Experiments.—Weigh accurately a small, clean, dry flask. Pipet into it 25 c.c. of urine and weigh again. From the data obtained calculate the specific gravity of the urine.

Determine the specific gravity of the same sample of urine by means of an ordinary clinical areometer. Compare the results obtained, and explain how to standardize a clinical areometer.

Collect a full twenty-four hour quantity of urine, and in it determine the following: Volume, specific gravity, acidity, total nitrogen, urea, ammonia, uric acid, creatinin, chlorids, phosphates, sulphates, ethereal sulphates. Test qualitatively for indican.

For two days eat no meat, fish, eggs, milk, cheese, peas, or beans, and only a little bread. Eat much butter, potatoes, vegetables, starch preparations, fruit, and candy. Repeat all the determinations with the second twenty-four hour quantity.

For two days eat all the meat products you can, and collect the two twenty-four hour quantities of urine. In the second twenty-four hour quantity determine all the factors enumerated above. Tabulate and compare the results obtained in the three series of analyses.

Take 15 g. of sodium bicarbonate in divided doses, collect the twenty-four hour urine, and estimate the ammonia and acidity. Compare with the normal.

Two days later, beginning in the morning, take 5 g. ammonium chlorid in the course of the day. Determine the ammonia and acidity.

Eat much sweetbread, kidney, or liver, for one day; collect the urine for the whole twenty-four hours, and estimate the uric acid. Compare with the uric acid figures previously obtained. Explain.

PART VII

BLOOD

1. **Hemoglobin Crystals.**—Place a drop of defibrinated rat blood on a slide, add a drop or two of water, mix, and cover with a cover-glass. Sketch the crystals which separate after a few minutes.

2. **Hemoglobin (Reduced Hemoglobin).**—Add to dilute blood a few drops of strong ammonium sulphid, or one or two drops of freshly prepared Stokes' reagent.

Examine spectroscopically.

Stokes' reagent is a 2 per cent. solution of ferrous ammonium sulphate in 3 per cent. tartaric acid, to which is added ammonia until a clear solution is obtained. The ammonia should be added only to the amount of reagent immediately needed.

Shake the solution of hemoglobin with air, and note the rapid change to oxyhemoglobin. Change the same solution of oxyhemoglobin to hemoglobin, and reverse two or three times, and note the facility with which hemoglobin takes up and loses oxygen.

3. **Carbon monoxid hemoglobin.**—Pass a current of illuminating gas through a dilute oxyhemoglobin solution for a minute, and filter. Note the change of color. Try the effect on the solution of (1) ammonium sulphid, (2) Stokes' reagent, (3) potassium ferrocyanid, (4) shaking with air. Note the stability of the compound.

Examine spectroscopically.

4. **Methemoglobin.**—Add to dilute defibrinated blood (1:15) two drops of a freshly prepared solution of sodium nitrite. Note the change. What is the effect produced by the addition of reducing agents?

5. Hematin.—Hemolyze a small quantity of blood and add dilute hydrochloric acid cautiously till a precipitate occurs.

Acidify strongly with hydrochloric acid. Note color (acid hematin). Then add sodium hydrate till strongly alkaline. Note color (alkaline hematin). To the alkaline solution add a few drops ammonium sulphid and warm gently. Note color (reduced hematin or hemochromogen).

6. Hemin Crystals—Teishmann's Test.—Place a bit of powdered dried blood on a glass slide, add a minute crystal of sodium iodid and two drops of glacial acetic acid. Cover with a cover-glass and warm gently over a flame until bubbles appear. Describe the crystals which separate.

7. Fibrinogen.—Allow about 6-8 volumes fresh blood to run from the animal into 1 volume of a 1 per cent. potassium oxalate solution (why?). Allow to stand over night in the cold room, and siphon off the clear plasma. With the solution so obtained make the following experiments:

Dilute 10 c.c. with 20 c.c. of distilled water and divide into 3 equal portions. To one add a little dilute (1 per cent.) calcium chlorid solution. To the second add a few drops of blood serum (why?). Place the three tubes in a beaker of water heated to 40° and observe the time of clotting.

PART VIII

MILK

Determine the specific gravity as in the case of urine.

1. Determination of Total Nitrogen.—Transfer 25 c.c. milk to a 100 c.c. volumetric flask. Fill to the mark with water, mix, and determine the total nitrogen in 1 c.c. Calculate the total protein content of the milk by multiplying its nitrogen with the factors 6.25.

2. Determination of Casein.—Transfer 50 c.c. of the diluted milk to another 100 c.c. flask, and carefully precipitate the casein by the addition of dilute acetic acid and gentle shaking. Make up to volume (100 c.c.) with water. Centrifuge a portion, and determine the nitrogen in 5 c.c. of the clear liquid.

Calculate the total nitrogen (and protein), making due allowance for the dilutions, and subtract from the total protein found in the preceding experiment. The difference represents casein.

3. Determination of Milk Sugar.—Transfer 5 c.c. of milk to a small flask or beaker. Add 20 c.c. of water and mix well. Fill the special 5 c.c. sugar buret, used in glucose titrations, with the diluted milk. Transfer to a large test tube 5 c.c. of 5.9 per cent. copper sulphate solution and 1 c.c. of saturated sodic carbonate solution. Shake for a moment, then add about 5 g. of the salt mixture used in glucose titrations. (p. 57.) Add 4.2 c.c. of diluted cow's milk or 2.8 c.c. of diluted mother's milk and boil gently for four minutes counting from the time of actual beginning boiling. At the end of four minutes add more milk (0.02 to 0.3 c.c.) unless it is apparent that the initial addition is enough. Boil one minute after each fresh addition.

In the sugar titration in the case of milk one can assume that cow's milk will contain a little less than 5 per cent. and that mother's milk may contain no more, but may go as high as 7 per

cent., hence with mother's milk it is not advisable to start with more than 2.8 c.c. for the first boiling period.

Calculation: 5 c.c. of the copper sulphate solution is reduced in about 5 minutes by 40.4 mg. of lactose. 4.04 times 5, the degree of dilution, or 20.2, divided by the volume of diluted milk used gives the per cent. of lactose in the milk.

4. Determination of Fat.—Measure out 17.6 c.c. of thoroughly mixed milk into a Babcock flask. Add 17 c.c. sulphuric acid (sp. gr. 1.82) and mix thoroughly, with gentle turning and shaking, until all the precipitated proteins have dissolved. Rotate in the centrifuge for 3 minutes. Add hot water up to the beginning of the graduations in the neck of the flask, and rotate for 1 minute. The graduations read in per cent. of fat.

PART IX

BONE

Weigh a piece of clean, raw bone on the laboratory scales. Immerse in about 10 times its weight of 10 per cent. hydrochloric acid in a flask. If any gas is evolved, determine what it is.

After 48 hours dilute the volume of the solution and what remains of the bone to a definite volume in a cylinder. Mix so as to get the solution uniform in composition.

Pipet out 25 c.c. of the solution, neutralize with sodic hydrate, using congo red paper as indicator, and determine the phosphates. Repeat.

Calculate the tricalcic phosphate corresponding to the phosphoric acid found.

Taking other portions of the original solution, demonstrate experimentally that all the calcium in the bone can not be precipitated together with the phosphoric acid present.

In what form is this excess of calcium present in bone?

Examine the insoluble substance left in the hydrochloric acid solution. What is the substance? Prepare a "gelatin" solution from it.

PART X

BILE

1. Character of Bile.—Determine the specific gravity, taste, odor, color, consistency, reaction, of the bile supplied.

Test for coagulable protein.

2. Bile Salts.—Mix 250 c.c. ox-bile with one-fourth its volume of bone-black, and evaporate nearly to dryness on the water-bath. Cool, transfer the residue to a flask, and extract with 200 c.c. of alcohol over night. Filter, and evaporate the filtrate to dryness on the water-bath. Dissolve the residue in absolute alcohol, and filter into a dry flask. Add anhydrous ether till permanent cloudiness develops. Place in the cold room to crystallize. Filter. Describe the crystals.

3. Pettenkofer's Test for Bile Salts.—Mix a little bile with 2 or 3 drops of 10 per cent. solution of cane sugar. Place in a test tube some concentrated sulphuric acid. Incline the tube containing the sulphuric acid, and pour the bile solution slowly down the side of the tube so that it forms a layer above the sulphuric acid.

4. Gmelin's Test for Bile Pigments.—Put 5 c.c. of nitric acid, containing some nitrous acid, in a test tube, and introduce on top of it (pipet) about 5 c.c. of diluted bile. Note what occurs. Study the delicacy of the reaction with very dilute solution of bile.

5. Test for Bile in Urine.—The presence of bile in human urine is usually indicated by its color and the color of the foam. In making the nitric acid test for albumin the presence of bile is also revealed by a series of colored rings (green, blue, violet, red, and yellowish-red).

A similar series of colors is occasionally obtained from urines which have been preserved with thymol. This is one of the objections to this otherwise excellent preservative.

To 10 c.c. of urine add a few drops of calcium chlorid solution and a few drops of 10 per cent. sodic hydrate. Filter. Remove the filter paper from the funnel, open it, and drop 1 or 2 drops of concentrated nitric acid on the sediment. In the presence of (human) bile the usual, characteristic series of colored rings is obtained.

SUPPLEMENT

URINE

Qualitative Test for Acetone in Urine.—Clinicians seldom differentiate between acetone and diacetic acid, and the "acetone tests" which they use are tests for diacetic acid rather than for acetone. (*See* p. 159.)

In the qualitative test for acetone, as for its quantitative determination, the acetone is first removed from the urine by means of an air current, just as in corresponding determinations (and tests) for ammonia.

In the large test tube used for the colorimetric determination of ammonia place first 5 c.c. of urine and 1-2 drops dilute acid (HCl or H_2SO_4). Then insert the rubber stopper carrying the absorption tube, etc., place the test tube in a beaker of lukewarm water (35-40° C.), and aspirate the volatile acetone by means of a moderately rapid air current into a test tube containing 5 c.c. distilled water and 5 c.c. Scott-Wilson reagent. If acetone is present, even if only in minute traces, the solution becomes turbid. If the amount of acetone obtained is extremely small the turbidity may not appear for 5-10 minutes.

The Scott-Wilson reagent for acetone, which is used for qualitative tests as well as for quantitative determinations, is most conveniently prepared as follows:

To 10 g. of mercuric cyanid dissolved in 600 c.c. of water add a cooled solution of 180 g. of sodium hydroxid in 600 c.c. of water. Transfer this mixture to a heavy walled glass jar, and to it add 2.9 g. of silver nitrate dissolved in 400 c.c. of water. The silver solution should be added in a slow stream, and the addition must be accompanied by constant and exceedingly vigorous stirring with a heavy glass rod. If properly made, the silver dissolves completely, giving a clear solution which is at once available for use. If the solution is turbid, it should be set aside to settle for three or four days and the clear supernatant liquid removed by means of a siphon.

In the clear reagent a new sediment gradually forms, so that the

solution deteriorates slowly and after a few months is not serviceable for quantitative work, though still good for qualitative tests.

Titration of Acetone and Preparation of Standard Acetone Solutions.—Standard solutions of iodin, sodium thiosulphate, and potassium permanganate are needed in this work, the latter being used only as a basis for making the other two accurate. .5 N permanganate solution may be used.

Iodin—Weigh roughly 10-12 g. of potassium iodid in a beaker and add 50 c.c. of water. Weigh out 6.4 g. iodin in a small beaker covered with a watch glass and add this to the potassium iodid solution. Stir until the iodin is dissolved and then transfer the resulting solution to a 500 c.c. volumetric flask. Dilute to the mark with water and mix.

Sodium Thiosulphate. Weigh out 24.85 g. of the salt ($Na_2S_2O_3 + 5H_2O$), dissolve in water, transfer to a 500 c.c. volumetric flask, fill to the mark with water, and mix.

The two solutions thus prepared should be approximately tenth normal. Their relative values are determined by titration as follows:

Pipet 20 c.c. of the iodin solution into a flask (capacity 500-600 c.c.) and add about 100 c.c. water. From a buret, run in the thiosulphate solution until the reddish-brown iodin color has faded to a faint straw yellow. Now add a few drops of starch solution (*see* p. 65) and continue the titration till the blue iodid of starch color disappears. The end point of this titration is very sharp.

The value of the thiosulphate solution is now determined as follows:

Weigh roughly 2 g. potassium iodid, transfer to a flask, and dissolve in about 150 c.c. water. Add 5 c.c. diluted hydrochloric acid (1-5) and 50 c.c. .05 N potassium permanganate solution.

The permanganate sets free an equivalent quantity of iodin according to the following equation:

$$2KMnO_4 + 10KI + 16HCl = 12KCl + 2MnCl_2 + 8H_2O + 5I_2$$

The iodin thus liberated is then titrated with the sodium thiosulphate solution in the same manner as the original iodin solution.

From the relative values of the iodin, the thiosulphate, and the permanganate solutions, the exact values of the first two (in terms of tenth normal solutions) are calculated.

Standard Stock Solution of Acetone.—Add about 1 c.c. of *pure* acetone (from the bisulphite compound) to water in a one liter volumetric flask, dilute to the mark, and mix. The titration of acetone with iodin is based on the fact that in alkaline solutions the acetone solution deteriorates slowly and after a few months is not serviceable for quantitative work, though still good for qualitative tests.

Titration of Acetone and Preparation of Standard Solutions. Standard solutions of iodine, sodium thiosulphate and potassium permanganate are needed in this work, the latter being used only as a basis for making the other two accurate. A $N/10$ permanganate solution may be used.

Iodine.—Weigh roughly 18 g. of potassium iodide in a beaker and add 50 c.c. of water. Weigh out 12.7 g. iodine in a small beaker covered with a watch glass and add this to the potassium iodide solution. Stir until the iodine is dissolved and then transfer the resulting solution to a 1000 c.c. volumetric flask. Dilute to the mark with water and mix.

Sodium Thiosulphate.—Weigh out 24.8 g. of the salt ($Na_2S_2O_3 + 5H_2O$), dissolve in water, transfer to a 1000 c.c. volumetric flask, fill to the mark with water, and mix.

The two solutions thus prepared should be approximately tenth normal. Their relative values are determined by titration as follows:

Pipet 20 c.c. of the iodine solution into a flask (preferably glass-stoppered) and add about 100 c.c. water. From a burette run in the thiosulphate solution until the reddish-brown iodine color has faded to a faint straw yellow. Now add a few drops of starch solution (see p. 68) and continue the titration till the blue color of starch iodide disappears. The end point of this titration is very sharp.

The value of the thiosulphate solution is now determined as follows:

Weigh roughly 1 g. potassium iodide, transfer to a flask and dissolve in about 150 c.c. water. Add 5 c.c. diluted hydrochloric acid (1:1) and 20 c.c. $N/10$ potassium permanganate solution.

The permanganate sets free an equivalent quantity of iodine according to the following equation:

$$2KMnO_4 + 10KI + 16HCl = 12KCl + 2MnCl_2 + 8H_2O + 5I_2.$$

The iodine thus liberated is then titrated with the sodium thiosulphate solution in the same manner as the previous iodine titration. From the relative values of the iodine, the thiosulphate and the permanganate solutions, the exact values of the first two (in terms of tenth normal solutions) are calculated.

Standard Stock Solution of Acetone.—Add about 1 c.c. of acetone (free from the bisulphite compound) to water in a one-liter volumetric flask, dilute to the mark, and mix. The titration of acetone with iodine is based on the fact that in alkaline solution the acetone is converted into iodoform. Several reactions are involved in this process:

Iodine is converted into hypo-iodite,

1. $I_2 + 2KOH = KOI + KI + H_2O$.

Hypo-iodite is then slowly converted into (useless) iodate,

2. $3KOI = KIO_3 + 2KI$.

The hypo-iodite converts acetone into iodoform and acetic acid,

3. $3KOI + CH_3COCH_3 = CH_3COCI_3 + 3KOH$.
4. $CH_3COCI_3 + KOH = CH_3COOK + CHI_3$

On acidifying, after the iodoform has been formed, the surplus iodin present as hypo-iodite (and iodate) is set free, and can then be titrated with the standard sodium thiosulphate solution as described above.

Each molecule of acetone uses up three molecules of hypo-iodite, and as each molecule of the latter is formed at the expense of two atoms of iodin, six atoms of iodin correspond to one molecule of acetone. One c.c. of .1 N iodin solution corresponds therefore to .968 mg. acetone. Because of the iodate formation a considerable excess must be added.

The titration of the acetone solution is carried out as follows:

Transfer 25 c.c. of the stock acetone solution to a flask, add 150-200 c.c. water, then 50 c.c. of the standardized iodin solution, and 10 c.c. strong sodic hydrate (40 per cent.). Let stand with occasional shaking for 5 minutes. Add 18 c.c. concentrated hydrochloric acid, and titrate the liberated excess of iodin with the standard thiosulphate solution.

If the standard solutions are exactly tenth normal, subtract the volume of thiosulphate employed from the volume of iodin solution taken, and multiply the remainder (in c.c.) with .968 to obtain the acetone content (in mg.).

Calculate the acetone content of the stock solution (in mg. per c.c.). Transfer to a distilling flask as much of it as contains exactly 50 mg. of acetone. Add water enough to make a volume of 500-600 c.c., and distill with vigorous cooling of the condenser. The receiver should be a large flask (750-1000 c.c.) containing about 250 c.c. approximately normal sulphuric acid. Boil for 20-30 minutes, or until at least 150 c.c. of distillate has gone over. Transfer this distillate to a volumetric (liter) flask and dilute to the mark with water. Ten c.c. of the acetone solution so obtained contains .5 mg. acetone. This solution, as well as the original stock solution, should be kept in a well stoppered bottle. The sulphuric acid present in the dilute standard acetone solution is added to prevent polymerization.

Preparation of Standard Acetone Solutions from the Acetone Bisulphite Compound.—A standard acetone solution can be prepared

without distillations from the "acetone sulphite" used in photography as follows:

Transfer 2.5 g. of the powder to a volumetric (1,000 c.c.) flask by means of a little water (50 c.c.), and fill up to the mark with dilute (1 in 5) hydrochloric acid. Transfer 25 c.c. of the solution to a flask. Add 20 c.c. tenth normal iodin, let stand for five minutes, and titrate the surplus iodin with tenth normal thiosulphate solution. This titration gives the SO_2 or the sodium bisulphite content.

To another 25 c.c. of the acetone solution add 50 c.c. tenth normal iodin, let stand five minutes, then add 10 c.c. strong sodic hydrate, followed after five minutes by 18 c.c. concentrated hydrochloric acid. Titrate the liberated iodin with thiosulphate. From the 50 c.c. of iodin taken subtract (a) the figure of the last thiosulphate titration and (b) the iodin corresponding to the SO_2. The remainder corresponds to the acetone. From the standardized stock solution prepare the more dilute standard solution (5 c.c. or 10 c.c. of which should contain exactly half a mg. of acetone).

If the "acetone sulphite" is not available, acetone sodium bisulphite is easily prepared by slowly adding (with stirring) two-thirds volume of ordinary acetone to one volume (100 or 200 c.c.) of saturated sodium bisulphite solution (freshly prepared and filtered). The precipitate should be freed as completely as possible from the mother liquor by filtering on a Buchner funnel with suction. Then wash rapidly two or three times with alcohol. Let dry in the open air for two or three days. Sieve to make the preparation uniform, and preserve in glass stoppered vessel.

Quantitative Determination of Acetone in Urine.—To about 1 c.c. of 10 per cent. sulphuric acid in a large test tube add enough urine (.5 to 5 c.c.) to give about .5 mg. of free acetone (.3-.7 mg.). Connect the test tube, as in ammonia determinations, with a second test tube containing 10 c.c. of fresh approximately 2 per cent. sodium bisulphite solution. Warm the first test tube to 35-40° C., as in the qualitative test for acetone and aspirate the acetone into the bisulphite solution by means of a moderate air current (time about 10 minutes). Transfer the sulphite-acetone solution to a 100 c.c. volumetric flask together with distilled water enough to make 50-60 c.c. To each of two other 100 c.c. flasks add 10 c.c. of the standard acetone solution containing .5 mg. acetone, add 10 c.c. of the 2 per cent. bisulphite solution, and dilute with distilled water to 50-60 c.c.

To each of the three flasks add 15 c.c. (clear) Scott-Wilson reagent, and immediately (before turbidity formation) dilute with without distillation from the "acetone sulphite" used in photography as follows:

Transfer 2.5 g. of the powder to a volumetric (1,000 c.c.) flask by means of a little water (50 c.c.), and fill up to the mark with dilute (1 in 5) hydrochloric acid. Transfer 25 c.c. of the solution to a flask. Add 50 c.c. tenth normal iodin, let stand for five minutes, and titrate the surplus iodin with tenth normal thiosulphate solution. This titration gives the SO_2 or the sodium bisulphite content.

To another 25 c.c. of the acetone solution add 50 c.c. tenth normal iodin, let stand five minutes, then add 10 c.c. strong sodic hydrate, followed after five minutes by 10 c.c. concentrated hydrochloric acid. Titrate the liberated iodin with thiosulphate. From the 50 c.c. of iodin taken subtract (a) the figure of the last thiosulphate titration and (b) the iodin corresponding to the SO_2. The remainder corresponds to the acetone. From the standardized stock solution prepare the more dilute standard solution (5 c.c. or 10 c.c. of which should contain exactly half a mg. of acetone).

If the "acetone sulphite" is not available, acetone sodium bisulphite is easily prepared by slowly adding (with stirring) two-thirds volume of ordinary acetone to one volume (100 or 200 c.c.) of saturated sodium bisulphite solution (freshly prepared and filtered). The precipitate should be freed as completely as possible from the mother liquor by filtering on a Buchner funnel, with suction. Then wash rapidly two or three times with alcohol. Let dry in the open air for two or three days. Sieve to make the preparation uniform, and preserve in glass stoppered vessel.

Quantitative Determination of Acetone in Urine.—To about 5 c.c. of 10 per cent. sulphuric acid in a large test tube add enough urine (1-5 c.c.) to give about .5 mg. of free acetone (.3-.7 mg.). Connect the test tube, as in ammonia determinations, with a second test tube containing 10 c.c. of fresh approximately 2 per cent. sodium bisulphite solution. Warm the first test tube to 50° C., as in the qualitative test for acetone and aspirate the acetone into the bisulphite solution by means of a moderate air current (time about 10 minutes). Transfer the sulphite-acetone solution to a 100 c.c. volumetric flask together with distilled water enough to make 50-60 c.c. To each of two other 100 c.c. flasks add 10 c.c. of the standard acetone solution, containing .5 mg. acetone, and 10 c.c. of the 2 per cent. bisulphite solution, and dilute with distilled water to 50-60 c.c.

To each of the three flasks add 15 c.c. (clear) Scott-Wilson reagent and immediately (before turbidity formation) dilute with distilled water to the mark, mix, and let stand for 12-15 minutes. Read the turbid contents of one of the standard acetone suspensions against itself in the Duboscq colorimeter. The best source of light for these comparisons is diffuse daylight coming through an opening (about 25 cm. square) cut through the shade of a (north side) window. The colorimeter metal screen must also be used. The instrument must be adjusted until 20 mm. of the two suspensions are equal. Now replace the contents of one of the colorimeter cups with the contents of the second standard suspension, and the other with the unknown acetone mercury suspension obtained from the urine, and make the turbidity comparison in the same manner as colorimetric comparisons—setting the standard at 20 mm.

Twenty multiplied by .5 and divided by the reading of the unknown (in mm.) gives the acetone content (in mg.) of the volume of urine taken for the analysis.

Qualitative Test for Diacetic Acid (in traces).—To 5 c.c. of urine in a test tube add 1-2 c.c. dilute acetic acid (10 per cent.) and a small crystal of sodium nitroprussid. Shake a few times to dissolve the salt, then add an excess of concentrated ammonia (2-3 c.c.), and mix. A violet color indicates diacetic acid.

Gerhardt's Ferric Chlorid Test for Diacetic Acid.—This test is useful for showing the presence in urine of relatively large amounts of diacetic acid. It is made as follows: To 5 c.c. of urine in a test tube add ferric chlorid solution (10 per cent.), drop by drop. At first a white precipitate of ferric phosphate is obtained, then, as the addition of the reagent is continued, a dark red color is produced if diacetic acid is present (in more than traces).

A number of substances used as drugs, such as salicylic acid, phenacetin, etc., give a similar reaction. If confusion due to such drugs is to be suspected, boil the deep red solution for 2-3 minutes. If the color is due to diacetic acid, it should disappear during the boiling and not reappear on cooling. The disappearance is due to the destruction by boiling of the unstable diacetic acid.

Quantitative Determination of Diacetic Acid (and Acetone).—Acetone urines contain from two or three to nine or ten times distilled water to the mark, mix, and let stand for 2–3 minutes. Read the turbid contents of one of the two standard suspensions against itself in the Duboscq colorimeter. The best source of light for these comparisons is diffuse daylight coming through an opening (about 25 cm square) cut through the shade of a north side window. The colorimeter metal screen must also be used. The instrument must be adjusted until, at 20 mm, the two suspensions are equal. Now replace the contents of one of the colorimeter cups with the contents of the second standard suspension, and the other with the unknown acetone mercury suspension obtained from the urine, and make the turbidity comparison in the same manner as colorimetric comparisons—setting the standard at 20 mm.

Twenty multiplied by 1 and divided by the reading of the unknown (in mm.) gives the acetone content (in mg.) of the volume of urine taken for the analysis.

Qualitative Test for Diacetic Acid (in traces).—To 5 cc. of urine in a test tube add 0.5 cc. dilute acetic acid (10 per cent.) and a small crystal of sodium nitroprusside. Shake a few times to dissolve the salt, then add an excess of concentrated ammonia (about 2 cc.) and mix. A violet color indicates diacetic acid.

Gerhardt's Ferric Chloride Test for Diacetic Acid.—This test is useful for showing the presence of relatively large amounts of diacetic acid and is made as follows: To 5 cc. of urine in a test tube add ferric chloride solution (10 per cent.) drop by drop. At first a white precipitate of ferric phosphate is obtained, then as the addition of the reagent is continued, a dark red color is produced if diacetic acid is present (in more than traces).

A number of substances used as drugs, such as salicylic acid, phenacetin, etc., give a similar reaction. If such drugs are to be suspected boil the original solution for five minutes. If the color is due to diacetic acid it should disappear during the boiling and not reappear on cooling. The disappearance is due to the destruction by boiling of the unstable diacetic acid.

Quantitative Determination of Diacetic Acid (and Acetone).—Acetone urines contain from two or three to nine or ten times as much aceto-acetic acid as acetone. In strictly fresh urines the latter proportions prevail; but the older the urine the greater becomes the relative proportion of acetone, because of the spontaneous decomposition of the aceto-acetic acid. Urines giving a strong ferric chlorid reaction usually contain more than .5 mg. of aceto-acetic acid per cubic centimeter, and must be diluted so that an appropriate fraction of 1 c.c. (of the original urine) can be taken for a determination.

The amount of urine taken should yield approximately .5 mg. of acetone (from .3 to .7 mg.). Transfer this amount of urine to a large test tube containing 1 c.c. of 10 per cent. sulphuric acid, and connect with a second test tube containing 10 c.c. of 2 per cent. sodium bisulphite solution. Immerse the test tube containing the urine in a beaker of boiling water and the second test tube in cold water, then pass through an *extremely* slow air current for ten minutes. Increase slightly the speed of the air current and continue the aspiration for another five minutes. The aceto-acetic acid plus acetone is thus transferred, in the form of acetone, to the bisulphite solution. Rinse the solution into a 100 c.c. volumetric flask, and determine the acetone exactly as in the determination of the performed acetone.

One mg. of acetone is equivalent to 1.8 mg. of aceto-acetic acid. From the "total acetone" of the 24-hour quantity of urine is subtracted the total preformed acetone, and the remainder multiplied by 1.8 gives the aceto-acetic acid.

Determination of Beta-oxybutyric Acid in Urine.—The method described below was at first thought by its authors (Folin and Denis) to give strictly all the beta-oxybutyric acid present in urine. But it now appears that the yield is only 85-95 per cent., just as in the original method of Shaffer.

The urine is diluted from 10-100 times, depending on how much of the substance is present. The ammonia content of the urine is the best index as to how much urine is required to yield the desired amount of beta-oxybutyric acid (1.5-3.5 mg.). The ferric chlorid test for diacetic acid is also helpful, but without considerable experience only a preliminary determination can give the desired information.

Measure diluted urine, equivalent to 1.5-3.5 mg. of beta-oxybutyric acid, into a 500 c.c. Kjeldahl flask, add a little dilute sulphuric acid (5 c.c.), and water enough to make a volume of about

150 c.c. Boil the mixture gently for ten minutes (to drive off the preformed acetone and the diacetic acid), then add to the solution (with a cylinder) 25 c.c. of a solution containing 1 per cent. potassium dichromate and 35 per cent. sulphuric acid, and connect the flask, in the usual manner, with a condenser by means of a specially treated rubber stopper.

The rubber stopper should be boiled twice for an hour in 10 per cent. sodic hydrate solution (or better, heated in an autoclave in the same solution for half an hour at 130-140° C.), and then thoroughly washed. It is also necessary to wrap the stopper thoroughly in tin foil during the distillation, so as to exclude the volatile sulphur impurities which otherwise are given off and interfere with the subsequent turbidity formation.

Distill very slowly, for one and one-half hours, collecting the distillate (about 100 c.c.) in another 500 c.c. Kjeldahl flask, previously charged with about 100 c.c. of water.

To the distillate add a small amount sodium peroxide (2 g.), and redistill by ordinary rapid boiling. Collect this final distillate in a 100 c.c. volumetric flask (or cylinder). About 80 c.c. should be obtained.

Dilute this distillate to the 100 c.c. mark with distilled water and mix. Transfer from 25 to 50 c.c. into a 100 c.c. volumetric flask, and determine the acetone content by the turbidity method, as in the case of the two preceding (acetone) determinations. No bisulphite is used in this case to hold the acetone, and none should therefore be added to the standard. Each milligram of acetone obtained corresponds to 1.78 mg. of beta-oxybutyric acid.

Shaffer's Short Method for the Determination of Beta-oxybutyric Acid.—To 50 c.c. of urine add 100 c.c. of water, then 50 c.c. of basic lead acetate solution (Goulard's Ext. U.S.P.), and stir. Add 50 c.c. approximately normal NaOH and stir again. Filter. A clear filtrate containing but traces of lead or glucose is usually obtained, even though the original urine contained considerable quantities of sugar. Traces of sugar do not interfere with the determination.

Introduce 50 c.c. of the filtrate into a 500 c.c. Kjeldahl flask previously marked at the level of 100 c.c. with a "glass pencil." Add 25 c.c. of water and 50 c.c. of half concentrated sulphuric [illegible]) and the mixture [illegible] gently [illegible] the [illegible] the peroxidised [illegible] and the distilled [illegible] the solution with a cylinder [illegible] c.c. of a solution containing [illegible] per cent. sodium dichromate and 25 per cent. sulphuric acid, and connect the flask in the usual manner, with a condenser, by means of a specially treated rubber stopper.

The rubber stopper should be boiled twice for an hour in 10 per cent. sodium hydroxide solution (or better, heated in an autoclave in the same solution for half an hour at [illegible] C.), and then thoroughly washed. It is also necessary to wrap the stopper thoroughly in tin foil during the distillation, so as to exclude the volatile sulphur impurities which otherwise are given off and interfere with the subsequent iodoform formation.

Distill very slowly, for one and one-half hours, collecting the distillate (about 250 c.c.) in another 500 c.c. Kjeldahl flask previously charged with about 200 c.c. of water.

To the distillate add a small amount of sodium peroxide (2 g.) and redistill by ordinary gentle heating. Collect the final distillate in a 250 c.c. volumetric flask (or cylinder). About 200 c.c. should be obtained.

Finally the distillate is [illegible] made up with distilled water and [illegible] 50 c.c. [illegible] into a 500 c.c. volumetric flask, and determine the acetone content by the iodometric method [illegible] the two [illegible] determinations. [illegible] is used in the [illegible] and more should [illegible] the [illegible]. Each milligram of acetone obtained corresponds to [illegible] of beta-oxybutyric acid.

Shaffer's Short Method for the Determination of Beta-oxybutyric Acid.—To 25 c.c. of urine add 100 c.c. of water, then 25 c.c. of basic lead acetate solution (Sp. gr. [illegible]) and [illegible]. Add 10 c.c. of ammonia [illegible] and [illegible] again. Filter. A clear filtrate containing but traces of lead or glucose is usually obtained, even though the original urine contained considerable quantities of sugar. Traces of sugar do not interfere with the determination.

Introduce 200 c.c. of the filtrate into a 500 c.c. Kjeldahl flask previously marked at the level of 500 c.c. with a glass pencil. Add 300 c.c. of water and 15 c.c. of half concentrated sulphuric acid. The latter, if freshly prepared by mixing with water (1 : 1), must be cooled before it is used.

Connect the Kjeldahl flask with a dropping funnel and with a condenser. Distill off about 25 c.c., collecting the distillate in another Kjeldahl flask.

The first distillate thus obtained contains the preformed acetone, as well as the acetone derived from the aceto-acetic acid of the urine. By adding to it 5 c.c. of strong alkali and redistilling, for 10 minutes, this acetone is obtained, in the second distillate, free from impurities, and can be titrated with iodin and thiosulphate.

After replacing the Kjeldahl flask used as a receiver with another one, the distillation of the urine filtrate is continued, while adding slowly (about 15 drops per 10 seconds) a .2 per cent. potassium bichromate solution.

During this distillation the volume in the distilling flask should be kept at approximately 100 c.c. (i.e., at the level indicated by the pencil mark). This is readily accomplished by regulating the speed of the distillation so that it just about equals the speed with which the bichromate solution is added. The speed of the oxidation is much greater with increasing concentration of sulphuric acid. With too great concentration of the acid, however, when the volume approaches a level of about 70 c.c., the oxybutyric acid is in part converted into crotonic acid, and thus escapes oxidation to acetone.

The bichromate solution is added only so fast as to maintain a *very slight* excess; the blue green color should largely predominate in the boiling mixture. Occasionally it may be necessary to interrupt the addition of bichromate for a few minutes, but the volume in the distilling flask should not be allowed to sink below 85 or 90 c.c. The addition of bichromate should be continued until (at the concentration of acid used) no more appears to be converted into the green chromium salt. From 50 c.c. to 100 c.c. bichromate solution (= .1 g.-.2 g. $K_2C_2O_7$) is usually required for each distillation. The addition (and distillation) lasts 20-30 minutes.

The distillate obtained must be redistilled, after the addition of 5 c.c. strong alkali and about 20 c.c. of 30 per cent. hydrogen peroxide. This final distillation need not last more than 10 minutes. The distillate thus obtained is titrated in the usual manner (p. 155) with iodin and thiosulphate. The yield of acetone obacid. The urine, if highly pigmented by phenols, with water (1 : 1), must be treated unless it is used.

Connect the Kjeldahl flask with a dropping funnel and with a condenser. Distill off about 250 c.c., collecting the distillate in another Kjeldahl flask.

The first distillate thus obtained contains the preformed acetone as well as the acetone derived from the aceto-acetic acid of the urine. By adding to it 5 c.c. of strong alkali and redistilling for 10 minutes, this acetone is obtained in the second distillate, free from impurities, and can be titrated with iodin and thiosulphate.

After replacing the Kjeldahl flask used as a receiver with another one, the distillation of the urine filtrate is continued, while adding slowly (about 40 drops per 10 seconds) 0.1 per cent potassium bichromate solution.

During this distillation the volume of the distilling flask should be kept at approximately 400 c.c. (the level being indicated by the pencil mark). This is readily accomplished by regulating the speed of the distillation so that it just about equals the speed with which the bichromate solution is added. The speed of the oxidation is much greater with increasing concentrations of sulphuric acid. With the above mixture it can be avoided, however, when the volume approaches several hundred c.c. above 400 c.c., the oxybutyric acid being converted into crotonic acid, and this escapes oxidation to acetone.

The bichromate solution, of which only 40 c.c. is used as a maximum, is added until excess; the blue-green color should largely predominate in the boiling mixture. Occasionally it may be necessary to interrupt the addition of bichromate for a few minutes. But the volume in the distilling flask should not be allowed to sink below 375 c.c. The addition of bichromate should be continued until the conversion of all β-oxybutyric acid is complete, as much as 600 c.c. being converted into acetone; as a rule, 20 to 50 c.c. of 0.1 per cent bichromate solution (= 1 c.c. 0.1 per cent $K_2Cr_2O_7$) is usually required for each distillation. The addition (and distillation) lasts 70–90 minutes.

The distillate obtained must be redistilled after the addition of 5 c.c. strong alkali and about 20 c.c. of 3 per cent hydrogen peroxide. This final distillation need not last more than 10 minutes. The distillate thus obtained is titrated in the usual manner (p. 159) with iodin and thiosulphate. The yield of acetone obtained is about 90 per cent. of the theoretical amount when working with solutions of pure beta-oxybutyric acid. A correction of 10 per cent. should therefore be added to the results obtained.

Slightly higher results (93-94 per cent.) may be obtained by a very slow addition of the bichromate, and a considerable prolongation of the distillation period, but since the theoretical amounts of acetone cannot be obtained the advantage so gained is doubtful.

Colorimetric Method for the Determination of Phenols in Urine.—The phosphotungstic phosphomolybdic reagent described in connection with the colorimetric determination of uric acid (p. 205) was originally devised as a reagent for phenols, and is serviceable for the determination of phenols in urinary filtrates from which the uric acid has been removed.

Transfer 10 c.c. of ordinary, or 20 of very dilute, urine to a 50 c.c. volumetric flask. Add acid silver lactate solution * (from 2 to 10 c.c.) until no more precipitate is obtained, then add a few drops of colloidal iron, and shake. Fill to the mark with distilled water, shake again, and filter. By means of this precipitation uric acid and traces of proteins are quantitatively removed. Transfer 25 c.c. of the filtrate to a 50 c.c. volumetric flask, and to it add a sufficient quantity of saturated sodium chlorid solution (containing 10 c.c. of strong hydrochloric acid per liter) to precipitate all the silver. Fill to the mark with distilled water and filter.

To determine "free" (non-conjugated) phenols, place 20 c.c. of this filtrate in a 50 c.c. flask, and treat with 5 c.c. of the phosphotungstic phosphomolybdic acid reagent and 15 c.c. of saturated sodium carbonate solution. After diluting to volume with lukewarm water (30-35° C.) and allowing to stand for twenty minutes, read the deep blue solution in a Duboscq colorimeter against a standard solution of phenol.

To determine total (free and conjugated) phenols, transfer 20 c.c. of the same filtrate to a large test tube, add ten drops of concentrated hydrochloric acid, and cover the test tube with a small funnel. Heat rapidly to boiling over a free flame, and then place in a boiling water-bath (usually a tall beaker) for ten minutes. At the end of this time remove the tube, cool, and trans-

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* This solution consists of a 5 per cent. silver lactate solution in 5 per cent. lactic acid.

fer the contents to a 100 c.c. volumetric flask. Add 10 c.c. of the phosphotungstic phosphomolybdic reagent and 25 c.c. of saturated sodium carbonate solution. Make up to volume, shake, and let stand for 20 minutes. Read against a standard solution of phenol.

The standard is a solution of pure phenol in .01 N HCl, containing .5 mg. of the former substance in 5 c.c. To 5 c.c. of the standard solution in a 100 c.c. flask add 10 c.c. of the reagent and 25 c.c. of saturated sodium carbonate solution. Fill up to the mark with water (at about 30° C.), and make the color comparison in the usual manner, setting the standard at 20 mm. As phenol is an exceedingly hygroscopic substance, it is necessary to standardize the solution by means of the iodometric titration.

This titration is carried out as follows: Make a phenol solution in .1 N HCl, containing 1 mg. of crystallized phenol per c.c. Transfer 25 c.c. of the phenol solution to a 250 c.c. flask, add 50 c.c. .1 N sodic hydrate, heat to 65° C., add 25 c.c. .1 N iodin solution, stopper the flask, and let stand at room temperature thirty to forty minutes. Add 5 c.c. of concentrated hydrochloric acid and titrate excess of iodin with .1 N sodium thiosulphate solution. One c.c. of .1 N iodin solution corresponds to 1.567 mg. of phenol. On the basis of the results, dilute the phenol solution so that 10 c.c. contains 1 mg. of phenol.

Because of the red precipitate in the solution it is rather difficult to see the end point of the titration. For those who have not had much experience it may be advisable to dilute the solution to a definite volume (after adding the hydrochloric acid), then to filter, and to titrate a portion of the filtrate as recommended by Sutton; with a little practice, however, the titration can be made without this procedure.

Quantitative Determination of Hippuric Acid in Urine.—In this method the hippuric acid is first hydrolyzed and the resulting benzoic acid is extracted with chloroform and the chloroform solution is titrated with standard alcoholic sodic hydrate.

Transfer 100 c.c. of urine to an evaporating dish, add 10 c.c. 5 per cent. sodic hydrate solution, and evaporate to dryness on the water-bath. Rinse the residue into a 500 c.c. Kjeldahl flask by means of 25 c.c. of water and 25 c.c. concentrated nitric acid. Add .2 g. copper nitrate, a couple of pebbles to prevent bumping, and boil very gently over a microburner for four and one-half

hours. During this boiling a miniature Hopkins' condenser (made from a large test tube) is kept within the neck of the boiling flask to prevent loss of benzoic acid which is volatile with steam.

After cooling rinse the condenser with 25 c.c. water, and transfer the contents of the flask to a separatory funnel (capacity 500 c.c.). Rinse the flask with 25 c.c. water, thus making the total volume in the separatory funnel 100 c.c.

Add to this solution 55 g. of ammonium sulphate, shake until dissolved, and extract with neutral (freshly washed) chloroform four times, using 50, 35, 25, and 25 c.c. of chloroform respectively. Collect the chloroform extracts in another separatory funnel, and wash this by shaking with 100 c.c. saturated solution of pure sodium chlorid, to each liter of which has been added .5 c.c. concentrated hydrochloric acid.

Draw off the chloroform which contains the benzoic acid into a dry flask, and titrate with a dilute standardized sodium alcoholate solution and 4-5 drops of phenolphthalein as indicator. The first distinct coloration diffusing through the whole liquid is taken as the end point without regard to subsequent fading.

The sodium ethylate solution is made by dissolving from 1.8 g. to 2.3 g. metallic sodium in absolute alcohol and diluting to a liter with absolute alcohol. It is standardized against chloroform solutions of benzoic acid.

One cubic centimeter of .1 N alcoholate corresponds to 1.22 mg. benzoic acid or 1.79 mg. hippuric acid.

Turbidity Method for the Determination of Albumin in Urine.— To about 75 c.c. of water in each of two 100 c.c. volumetric flasks add 5 c.c. of a 25 per cent. solution of sulphosalicylic acid. To one flask add 5 c.c. of a standard protein solution, prepared as described below, and containing 10 mg. of albumin. To the other add the albuminous urine 1 c.c. at a time (by means of an Ostwald pipet) until the turbidity obtained seems to be reasonably near that of the standard. Fill the two flasks up to the mark with water, cautiously inverting a few times to secure mixing. The standard must invariably first be read against itself to secure the adjustment of the colorimeter (and of the eye). Then replace the contents of one of the Duboscq colorimeter cups by

the suspension of the unknown, and make the turbidity comparison in the usual manner.

Set the standard containing 10 mg. of protein at 20 mm. The unknown must not read less than 10 nor more than 30 mm. Dividing 200 by the product of the reading of the unknown and the number of cubic centimeters of urine taken, gives the albumin in milligrams per cubic centimeter of urine.

It is very important not to shake the albuminous suspensions in the volumetric flasks because of the tendency of the precipitate to agglutinate. The preliminary mixing must therefore be accomplished by means of a few gentle inversions.

The standard protein solution is prepared from fresh blood serum free from hemoglobin. For the preparation of this serum either slaughter house or normal human blood may be used. The so-called blood serum sold for the preparation of bacteriological culture media should be avoided, as it is usually several days old and is frequently partially decomposed. The dried preparations of "blood albumin" listed by chemical dealers are also not satisfactory for the preparation of standard solutions. To prepare the standard, dilute 25-35 c.c. of serum with a 15 per cent. solution of chemically pure sodium chlorid to about 1500 c.c. Mix and filter. By means of nitrogen determinations ascertain the protein content of the filtrate (protein = N X 6.25) and on the basis of the figure obtained, dilute the solution with 15 per cent. sodium chlorid solution so that it contains 2 mg. of protein per cubic centimeter. Sodium chlorid in the concentration mentioned is fairly effective as a preservative. Nevertheless it is best to saturate the standard albumin solution with chloroform (20 c.c.).

The above method is not applicable to urines which are very deeply colored with blood or bile pigments. The method is of course applicable to other albuminous fluids than urine, as, for example, exudates, transudates, and the cerebrospinal fluid.

Gravimetric Method for the Determination of Albumin in Urine.—The method is as follows: Pipet 10 c.c. of urine into an ordinary conical centrifuge tube, which has been previously weighed; add 1 c.c. of 5 per cent. acetic acid, and let stand for fifteen minutes in a beaker of boiling water. At the end of this time remove the tube from the water-bath and centrifuge for a few minutes. Pour off the supernatant liquid, stir up the precipitate in the tube with about 10 c.c. of boiling .5 per cent. acetic acid, and again centrifuge. Remove the supernatant liquid from the precipitate

in the tube and wash once more, this time with 50 per cent. alcohol. After centrifuging and pouring off the supernatant alcohol, place the tube for two hours in an air bath at 100-110°, then cool in a desiccator, and weigh.

McCrudden's Method for the Determination of Calcium and Magnesium in Urine (*J. Biol. Chem.*, 7, 82 and 10, 187).—If the urine is alkaline, make it neutral or slightly acid to litmus. Filter. Transfer 200 c.c. of the filtered urine to a small flask. Make just alkaline with concentrated ammonium hydrate and then just acid with (concentrated) hydrochloric acid. The cloud of phosphates forming in alkaline urine may be used as a guide in the process of acidifying the urine. Cool for a few minutes in running water. Add 10 drops of concentrated hydrochloric acid and 10 c.c. of 2.5 per cent. oxalic acid. Now add 8 c.c. of 20 per cent. sodic acetate solution, stopper, and shake vigorously and continuously for about ten minutes. Filter on a small ash free filter paper and wash free from chlorids with .5 per cent. ammonium oxalate solution. Transfer the filter and precipitate to a weighed platinum crucible, dry over a small flame, and then heat in the blast lamp to constant weight, thus transforming the calcium oxalate to calcium oxid. Cool in a desiccator and weigh.

In the combined filtrate and washwater the magnesium is determined as follows: Transfer the filtrate to a large porcelain dish, add 20 c.c. concentrated nitric acid, and boil down almost to dryness. When the residue is nearly dry and no more nitrous fumes are given off, add 10 c.c. concentrated hydrochloric acid and again boil down nearly to dryness. Dilute with water to a volume of almost 80 c.c., and with constant stirring add ammonia, drop by drop, until the mixture is alkaline to litmus paper. Then add 25 c.c. dilute ammonia (sp. gr. .96) slowly and with stirring, and set aside over night in a cool place. Filter on a small filter paper, and wash the precipitate with a dilute solution of alcohol and ammonia (1 volume of alcohol and 1 volume dilute ammonia mixed with 3 volumes of water). Wash until the filtrate is free from chlorids. Dry the filter and ignite in a weighted platinum crucible. Cool and weigh. The residue is $Mg_2P_2O_7$.

Method for the Determination of Sodium and Potassium in Urine.—Transfer 50 c.c. of urine to a platinum dish (capacity about 250 c.c.), evaporate to dryness, and then heat the residue, at first

very cautiously, over a radial burner. Continue the heating at a barely perceptible dull red heat for half one hour. Cool. Moisten the residue with ~~20 c.c.~~ distilled water, evaporate to dryness, and heat as before for another hour. To the residue, which now should contain very little carbon, add 10 ~~50~~ c.c. water and 5-6 drops concentrated hydrochloric acid. The mineral constituents are thus brought into solution. Add an excess o~~f saturate~~d barium hydroxid s~~olution~~ (i.e., to a distinctly alkaline reaction), heat to boiling, and filter ~~on a Gooch crucible~~. Wash with hot water. The filtrate should now be substantially free from calcium, magnesium, phosphoric acid, and sulphuric acid, but does contain barium in addition to the sodium and potassium. Precipitate the barium by ~~passing washed carbonic acid~~ through the solution. Filter on another Gooch crucible and wash with a little cold water. Render the filtrate slightly acid to methyl orange (one drop), and evaporate to dryness in a previously weighed platinum dish. Heat the residue very gradually and carefully to a dull red heat for 10 minutes. Cool in a desiccator and weigh. The increase in weight gives the sodium and potassium as chlorids.

Dissolve the residue in a very small quantity of water and add a few drops dilute hydrochloric acid. Then add 10 per cent. chlorplatinic acid solution (4-5 times as much H_2PtCl_6 as the combined weight of the chlorids present), and evaporate at medium temperature, about 75° C., until the residue looks dry. Now add 95 per cent. alcohol, filter on a weighed Gooch crucible, and wash several times with 95 per cent. alcohol. Dry at 110° and weigh. The potassium chlorplatinate thus obtained multiplied by the factor .3056 gives the corresponding weight of potassium chlorid. The sodium chlorid is then obtained by subtracting the weight of the potassium chlorid from the weight of the combined chlorids.

In connection with this determination there are two fruitful sources of error: contamination of the chlorplatinate precipitate with ammonia (which, as ammonium chlorplatinate, gives too high results for potassium), and overheating during the ashing (which causes volatilization of the sodium chlorid). Loss of chlorids through overheating may be avoided by placing the platinum dish containing the dried urine on fragments of clay plate, or pieces of a broken evaporating dish, contained in a shallow iron dish (about 20 cm. in diameter) which is heated by means of a large size radial burner.

Preparation of Protein-Free Blood Filtrates.—(*J. Biol. Chem.*, 38, 81, 1919). The blood filtrate, the preparation of which is described below, is suitable for the determination of non-protein nitrogen, urea, uric acid, creatinin, creatin and sugar.

The blood should be collected over finely powdered potassium oxalate, about 20 mg. for 10 c.c. of blood. It is important not to use unnecessarily large amounts of oxalate because the excess makes the complete coagulation of the proteins more difficult and also interferes more or less with the uric acid precipitation.

Reagents required for the precipitation of the proteins:

1. A 10 per cent. solution of sodium tungstate. Some sodium tungstates, though labeled c.p., are not serviceable for this work. They usually contain too much sodium carbonate. The c.p. sodium tungstate made by the Primos Chemical Company, Primos, Pa., is satisfactory.

2. A two-thirds normal sulphuric acid solution, 35 g. of concentrated c.p. sulphuric acid diluted to a volume of 1 liter, will usually be found to be correct; but it is advisable, indeed necessary, to check it up by titration. The two-thirds normal acid is intended to be equivalent to the sodium content of the tungstate so that when equal volumes are mixed substantially the whole of the tungstic acid is set free without the presence of an excess of sulphuric acid. The tungstic acid set free is nearly quantitatively taken up by the proteins and the blood filtrates obtained are therefore only slightly acid to congo red paper.

Transfer a measured quantity (5 to 15 c.c.) of oxalated blood to a flask having a capacity of fifteen to twenty times that of the volume taken. Lake the blood with seven volumes of water. Add one volume of 10 per cent. solution of sodium tungstate ($Na_2WO_4,2H_2O$) and mix. Add from a graduated pipet or buret, slowly and with shaking, one volume of two-thirds normal sulphuric acid. Close the mouth of the flask with a rubber stopper and shake. If the conditions are right, hardly a single air bubble will form as a result of the shaking. Let stand for 5 minutes; the color of the coagulum gradually changes from bright red to dark brown. If this change in color does not occur, the coagulation is incomplete, usually because too much oxalate is present. In such an emergency the sample may

be saved by adding 10 per cent. sulphuric acid, one drop at a time shaking vigorously after each drop, and continuing until there is practically no foaming and until the dark brown color has set in.

Pour the mixture on a filter large enough to hold it all. This filtration should be begun by adding only a few c.c. of the mixture down the double portion of the filter paper and withholding the remainder until the whole filter has been wet. Then the whole of the mixture is poured on the funnel and covered with a watch glass. If the filtration is made as described the very first portion of the filtrate should be clear as water and no re-filtering is necessary.

It will be noted that the precipitation is not made in volumetric flasks. By the process described 6 or 7 or 11 or 12 c.c. of blood can be used, whereas with volumetric flasks one is compelled to use 5, 10 or 20 c.c., because flasks suitable for other volumes are not available. Special graduated "blood pipets," made by the Emil Greiner Co., New York, are very useful for the measurement of the blood, the tungstate and the acid.

The protein blood filtrates are not acid enough to prevent bacterial decomposition. If the filtrates are to be kept for any length of time, more than two days, some preservative, a few drops of toluene or xylene should be added.

Determination of Non-protein Nitrogen.—For the digestion of 5 c.c. of blood filtrate it is not necessary to use more than one-half c.c. of the phosphoric-sulphuric acid mixture described on p. 27. Dilute 50 c.c. of the acid mixture with 50 c.c. of water and keep well protected to prevent the absorption of ammonia. Use 1 c.c. for each digestion.

The digestion is most conveniently made in ignition test tubes (Pyrex, 200 mm. x 25 mm.) which have been graduated at 35 c.c. and at 50 c.c. Such test tubes can be obtained from the Emil Greiner Co., New York.

Transfer 5 c.c. of the blood filtrate to such a test tube. The test tube should either be dry or rinsed with alcohol to reduce the danger of bumping. Add 1 c.c. of the diluted acid mixture and a quartz pebble. Boil vigorously over a micro burner until the characteristic dense fumes begin to fill the tube. This will happen in from 3 to 7 minutes, depending on the size of the flame. When the test tube is nearly full of fumes reduce the flame

NPN – 28.8 mg/100 cc
Urea – 19.3 mg/100 or 14 gm. Urea N

sharply so that the speed of the boiling is reduced almost to the vanishing point. Cover the mouth of the test tube with a watch glass. Continue the gentle heating for 2 minutes, counting from the time the test tube became filled with fumes. If the oxidations are not visibly finished at the end of two minutes the heating must be continued until the solution is nearly colorless. Usually the solution becomes colorless at the end of 20 to 40 seconds. At the end of 2 minutes remove the flame and allow the digestion mixture to cool for 70 to 90 seconds. Then add 15 to 25 c.c. of water. Cool further approximately to room temperature and then fill to the 35 c.c. mark with water. Add 15 c.c. of Nessler's solution (p. 203). Insert a clean rubber stopper and mix. If the solution is turbid, centrifuge a portion before making the color comparison with the standard.

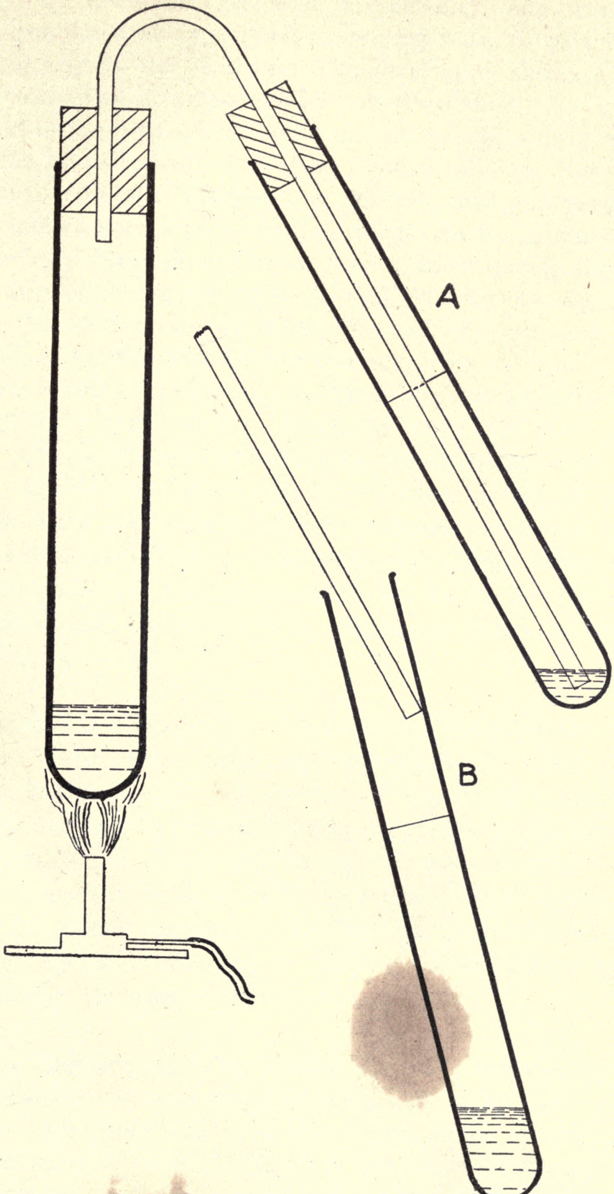
The standard most commonly required is 0.3 mg. of N. Add 3 c.c. of the standard ammonium sulphate solution (containing 1 mg. of N per 10 c.c.) to a 100 c.c. volumetric flask. Add to it 2 c.c. of the phosphoric sulphuric acid mixture, to balance the acid in the test tube; dilute to about 60 c.c. and add 30 c.c. of Nessler's solution. The unknown and the standard should be Nesslerized simultaneously.

Calculation.—If the standard is set at 20 mm. for the color comparison, 20 divided by the reading and multiplied by 0.3 gives the non-protein nitrogen in 1 c.c. of blood, because 0.5 c.c. (the amount of blood represented in 5 c.c. of the blood filtrate) Nesslerized at a volume of 50 c.c. is equivalent to 1 c.c. Nesslerized at a volume of 100 c.c.

The non-protein nitrogen per 100 c.c. of blood is therefore 20 divided by the reading and multiplied by 30 (0.3 times 100).

If the standard containing 0.5 mg. N is used the calculation becomes 20, divided by R, times 50.

DETERMINATION OF UREA.—Transfer 5 c.c. of the tungstic acid blood filtrate to a Pyrex ignition tube (200 x 25 mm.). This test tube must be rinsed with nitric acid and then with water if it has contained Nessler Solution. Add 2 drops of buffer mixture (p. 107) and then introduce 1 c.c. of urease solution (p. 107). Immerse the test tube in warm water, 40 to 55° C., and leave it there for 5 minutes, or let stand at room temperature for 15 minutes.



A, AT BEGINNING; B, TOWARD END OF DISTILLATION

The ammonia formed from the urea is most conveniently obtained by distillation, without a condenser, and using a test tube graduated at 25 c.c. and containing 2 c.c. of 0.05 N hydrochloric acid as the receiver. The illustration shows a compact and convenient arrangement for this distillation.

Add to the urease blood filtrate a dry pebble, a drop or two of paraffin oil and 2 c.c. of saturated borax solution. Insert firmly the rubber stopper carrying the delivery tube and receiver and then boil at a moderately fast, uniform rate for 4 minutes. The size of the flame should never be cut down during the distillation, nor should the boiling be so brisk that the emission of steam from the receiver begins before the end of 3 minutes. At the end of 4 minutes slip off the receiver from the rubber stopper and let it rest in a slanting position while the distillation is continued for 1 more minute. Rinse the lower end of the delivery tube with a little water and cool the distillate with running water and dilute to about 20 c.c. Transfer 0.3 mg. N (3 c.c. of the standard ammonium sulphate solution) to a 100 c.c. volumetric flask and dilute to about 75 c.c. Nesslerize, using 10 c.c. of Nessler's Solution for the Standard, and 2.5 c.c. for the unknown in the test tube. Dilute both to volume and make the color comparison.

Calculation.—Divide 20 (the height of the standard in mm.) by the colorimetric reading and multiply by 15. This gives the urea nitrogen in mgs. per 100 c.c. of blood. In explanation of this calculation it is to be noted that the unknown representing 0.5 c.c. of blood, is Nesslerized at 25 c.c., whereas in the case of the non-protein nitrogen it is Nesslerized at a volume of 50 c.c. The same colorimetric reading therefore represents only one-half as much nitrogen in the urea determination as in the non-protein nitrogen determination.

Urea Determination by Means of the Autoclave.—When a large number of urea determinations are to be made or when creatin determinations are also made, it is sometimes convenient to decompose the urea of the blood filtrate by heating under pressure. To 5 c.c. of the blood filtrate in a large test-tube add 1 c.c. of normal hydrochloric acid, cover with tin foil and heat to 150° for 10 minutes. Distil off the ammonia ex-

actly as in the preceding process, except that 2 c.c. of 10 per cent. sodium carbonate must be substituted for the borax, because of the added hydrochloric acid.

Aeration Process in Urea Determination.—The removal of the ammonia formed from the blood urea by urease, or by heating under pressure, can, of course, be driven into the receiver by an air current plus an alkali, instead of by the distillation process described above. The aeration process gives perfectly reliable results, if a good air current is available.

To the decomposed blood filtrate in a large test tube add a little paraffin oil and 1 or 2 c.c. of 10 per cent. sodium hydroxid. Connect with a smaller test tube, marked at 25 c.c., and containing 2 c.c. of 0.5 N hydrochloric acid. The connection is made as in the macro aeration process (*see* p. 91). Pass the air current through rather slowly for 1 minute and then nearly as fast as the apparatus can stand for 10 to 15 minutes. Rinse the connecting tube; dilute the contents of the receiver to 20 c.c., add 2.5 c.c. of Nessler Solution, dilute to the 25 c.c. mark, and make the color comparison in the usual manner.

DETERMINATION OF PREFORMED CREATININ.—Transfer 25 (or 50) c.c. of a saturated solution of purified picric acid to a small, clean flask, add 5 (or 10) c.c. of 10 per cent. sodium hydroxid, and mix. Transfer 10 c.c. of blood filtrate to a small flask or to a test tube, transfer 5 c.c. of the standard creatinin solution described below to another flask, and dilute the standard to 20 c.c. Then add 5 c.c. of the freshly prepared alkaline picrate solution to the blood filtrate, and 10 c.c. to the diluted creatinin solution. Let stand for 8 to 10 minutes and make the color comparison in the usual manner, never omitting first to ascertain that the two fields of the colorimeter are equal when both cups contain the standard creatinin picrate solution. The color comparison should be completed within 15 minutes from the time the alkaline picrate was added; it is therefore never advisable to work with more than three to five blood filtrates at a time

When the amount of blood filtrate available for the creatinin determination is too small to permit repetition, it is of course advantageous or necessary to start with more than one standard. If a high creatinin should be encountered unexpectedly without

several standards ready, the determination can be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution—using for such dilution a picrate solution first diluted with two volumes of water—so as to preserve equality between the standard and the unknown in relation to the concentration of picric acid and sodium hydroxid.

One standard creatinin solution, suitable both for creatinin and for creatinin determinations in blood, can be made as follows: Transfer to a liter flask 6 c.c. of the standard creatinin solution used for urine analysis (which contains 6 mg. of creatinin); add 10 c.c. of normal hydrochloric acid, dilute to the mark with water, and mix. Transfer to a bottle and add four or five drops of toluene or xylene. 5 c.c. of this solution contain 0.03 mg. of creatinin, and this amount plus 15 c.c. of water represents the standard needed for the vast majority of human bloods, for it covers the range of 1 to 2 mg. per 100 c.c. In the case of unusual bloods representing retention of creatinin, take 10 c.c. of the standard plus 10 c.c. of water, which covers the range of 2 to 4 mg. of creatinin per 100 c.c. of blood; or 15 c.c. of the standard plus 5 c.c. of water by which 4 to 6 mg. can be estimated. By taking the full 20 c.c. volume from the standard solution at least 8 mg. can be estimated; but when working with such blood it is well to consider whether it may not be more advantageous to substitute 5 c.c. of blood filtrate plus 5 c.c. of water for the usual 10 c.c. of blood filtrate.

Calculation.—The reading of the standard in mm. (usually 20) multiplied by 1.5, 3, 4.5, or 6 (according to how much of the standard solution was taken), and divided by the reading of the unknown, in m.m., gives the amount of creatinin, in mg. per 100 c.c. of blood. In connection with this calculation it is to be noted that the standard is made up to twice the volume of the unknown, so that each 5 c.c. of the standard creatinin solution, while containing 0.03 mg., corresponds to 0.015 mg. in the blood filtrate.

DETERMINATION OF CREATIN PLUS CREATININ.—Transfer 5 c.c. of blood filtrate to a test tube graduated at 25 c.c. These test tubes are also used for urea and for sugar determinations. Add 1 c.c. of normal hydrochloric acid. Cover the mouth of the test tube with tin-foil and heat in the autoclave to 130° C. for 20 minutes or, as for the urea hydrolysis, to 155° C.

for 10 minutes. Cool. Add 5 c.c. of the alkaline picrate solution and let stand for 8 to 10 minutes, then dilute to 25 c.c. The standard solution required is 10 c.c. of creatinin solution in a 50 c.c. volumetric flask. Add 2 c.c. of normal acid and 10 c.c. of the alkaline picrate solution and after 10 minutes standing dilute to 50 c.c. The preparation of the standard must of course have been made first so that it is ready for use when the unknown is ready for the color comparison. The height of the standard, usually 20 mm., divided by the reading of the unknown and multiplied by 6 gives the "total creatinin" in mg. 100 c.c. blood.

In the case of uremic bloods containing large amounts of creatinin 1, 2, or 3 c.c. of blood filtrate, plus water enough to make approximately 5 c.c., are substitutes for 5 c.c. of the undiluted filtrate.

The normal value for "total creatinin" given by this method is about 6 mg. per 100 c.c. of blood.

Determination of Uric Acid in Blood.—*Solutions Required for Uric Acid Determinations.*

1. The standard uric acid sulphite solution already described (p. 115.)

2. A 10 per cent. sodium sulphite solution.

3. A 5 per cent. sodium cyanid solution, to be added from a buret.

4. A 10 per cent. solution of sodium chlorid in 0.1 normal hydrochloric acid.

5. The uric acid reagent prepared according to Folin and Denis (*see* p. 207). A still stronger reagent is obtained by heating the sodium tungstate (100 gm.) and the phosphoric acid (80 c.c.) plus water (700 c.c.) for 24 hours, instead of 2 hours; but the advantage gained, about 20 per cent., is not needed. Dilute the solution to 1 liter.

6. A solution of 5 per cent. silver lactate in 5 per cent. lactic acid.

To 10 c.c. of blood filtrate in each of two centrifuge tubes add 2 c.c. of a 5 per cent. solution of silver lactate in 5 per cent. lactic acid, and stir with a very fine glass rod. Centrifuge; add a drop of silver lactate to the supernatant solution, which should be almost perfectly clear and should not become turbid when the last drop of silver solution is added. Remove the supernatant

liquid by decantation as completely as possible. Add to each tube 1 c.c. of a solution of 10 per cent. sodium chlorid in 0.1 normal hydrochloric acid and stir thoroughly with the glass rod. Then add 5 to 6 c.c. of water, stir again, and centrifuge once more. By this chlorid treatment the uric acid is set free from the precipitate. Transfer the two supernatant liquids by decantation to a 25 c.c. volumetric flask. Add 1 c.c. of a 10 per cent. solution of sodium sulphite, 0.5 c.c. of a 5 per cent. solution of sodium cyanid, and 3 c.c. of a 20 per cent. solution of sodium carbonate. Prepare simultaneously two standard uric acid solutions as follows:

Transfer to one 50 c.c. volumetric flask 1 c.c. and to another 50 c.c. flask 2 c.c. of the standard uric acid sulphite solution described above. To the first flask add also 1 c.c. of 10 per cent. sodium sulphite solution. Then add to each flask 4 c.c. of the acidified sodium chlorid solution, 1 c.c. of the sodium cyanid solution, and 6 c.c. of the sodium carbonate solution. Dilute with water to about 45 c.c. When the two standard solutions and the unknown have been prepared as described they are ready for the addition of the uric acid reagent. Add 0.5 c.c. of this reagent to the unknown and 1 c.c. to each of the standards, and mix. Let stand for 10 minutes, fill to the mark with water, mix, and make the color comparison.

Calculation.—In connection with the calculation it is to be noted (*a*) that the blood filtrate taken corresponds to 2 c.c. of blood, (*b*) that the standard is diluted to twice the volume of the unknown, and (*c*) that the standard used contains 0.1 or 0.2 mg. of uric acid. The blood filtrate from blood containing 2.5 mg. of uric acid will be just equal in color to the weaker standard. 20 times 2.5 divided by the reading of the unknown gives, therefore, the uric acid content of the blood when the weaker standard is set at 20 mm.

The two standards recommended were adopted on the basis of the experience gained from the analysis of more than 150 different samples of human blood. The uric acid may sink to as low as 1 mg. of uric acid per 100 c.c. of blood. It seems hardly worth while to prepare a third and weaker standard regularly in order to provide for such low acid values. A standard corresponding to the color obtained from 1.25 mg. of uric acid per 100 c.c. of blood can be prepared within a couple of minutes as follows: Transfer 1 c.c. of 10 per cent. sulphite solu-

tion, 3 c.c. of 20 per cent. sodium carbonate, 2 c.c. of the acidified sodium chlorid, 0.5 c.c. of the sodium cyanid solution, and 25 c.c. of the weaker one of the two regular standard solutions already on hand. Dilute to 50 c.c. and mix. Or, simply add 5 c.c. of 20 per cent. sodium carbonate to 25 c.c. of the regular weaker standard, and dilute to 50 c.c.

If a low uric acid value is expected, an alternate procedure is to dilute the unknown to a final volume of 10 c.c. with corresponding reduction in the amount of the reagents used.

Special attention should perhaps be called to one small yet essential variation in the process for developing the blue uric acid color, a variation made necessary by the use of sodium sulphite. The uric acid reagent must invariably be added after, and not before, the addition of the sodium carbonate, because in acid solution the sulphite will itself give a blue color with phosphotungstic acid.

New Method for Determination of Sugar in Blood.—*Solutions needed:*

1. *Saturated sodium carbonate solution.*

2. *Standard Sugar Solution.*—Dissolve 1 gm. of pure anhydrous dextrose in water and dilute to a volume of 100 c.c. Mix, add a few drops of xylene or toluene, and bottle. If pure dextrose is not available, a standard solution of invert sugar made from cane sugar is equally useful. Transfer exactly 1 gm. of cane sugar to a 100 c.c. volumetric flask; add 20 c.c. of normal hydrochloric acid and let the mixture stand over night at room temperature (or rotate the flask and contents continuously for 10 minutes in a water bath kept at 70° C.). Add 1.68 gm. of sodium bicarbonate and about 0.2 gm. of sodium acetate, to neutralize the hydrochloric acid. Shake a few minutes to remove most of the carbonic acid and fill to the 100 c.c. mark with water. Then add 5 c.c. more of water (1 gm. of cane sugar yields 1.05 gm. of invert sugar) and mix. Transfer to a bottle; add a few drops of xylene or toluene, shake well, and stopper tightly. The stock solution made in either way keeps indefinitely. Dilute 5 c.c. to 500 c.c., giving a solution 10 c.c. of which contain 1 mg. of dextrose or invert sugar. Add some xylene. Use 2 c.c. for each determination.

3. *Alkaline Copper Solution.*—Dissolve 40 gm. of anhydrous

sodium carbonate in about 400 c.c. of water and transfer to a liter flask. Add 7.5 gm. of tartaric acid and when the latter has dissolved add 4.5 gm. of crystallized copper sulphate; mix, and make up to a volume of 1 liter. If the carbonate used is impure, a sediment may be formed in the course of a week or so. If this happens, decant the clear solution into another bottle.

4. *Phosphotungstic-phosphomolybdic Acid.*—Transfer to a large flask 25 gm. of molybdenum trioxid (MoO_3) or 34 gm. of ammonium molybdate $(NH_4)_2(MoO_4)$; add 140 c.c. of 10 per cent. sodium hydroxid and about 150 c.c. of water. Boil for 20 minutes to drive off the ammonia (molybdic acid sometimes contains large amounts of ammonia as impurity). Add to the solution 100 gm. of sodium tungstate, 50 c.c. of 85 per cent. phosphoric acid, and 100 c.c. of concentrated hydrochloric acid. Dilute to a volume of 700 to 800 c.c.; close the mouth of the flask with a funnel and watch-glass. Boil gently for not less than 4 hours, adding hot water from time to time to replace that lost during the boiling. Cool and dilute to 1 liter. This solution is identical with the phenol reagent of Folin and Denis. For use in connection with the determination of blood sugar dilute 1 volume (100 c.c.) of the reagent with one-half volume (50 c.c.) of water and one-half volume (50 c.c.) of concentrated hydrochloric acid.

The determination of blood sugar is carried out as follows: Heat a beaker of water to vigorous boiling. Transfer 2 c.c. of the tungstic acid blood filtrate to a test-tube (20 m. × 200 mm.) graduated at 25 c.c. The graduated test-tubes used as receivers when distilling off the ammonia in urea determinations (p. 185) are suitable for this work. Transfer 2 c.c. of the dilute standard sugar solution to another similar test-tube. Add to each tube 2 c.c. of the alkaline copper tartrate solution. Heat in the boiling water for 6 minutes. Remove the test-tubes and add at once (without cooling), preferably from a graduated pipet, 1 c.c. of the strongly acidified and diluted phenol reagent. This should be done as nearly simultaneously as possible; it is not advisable to use one standard for a set of more than four determinations. The purpose of the added hydrochloric acid in the reagent is to dissolve the cuprous oxide. Mix, cool, and add 5 c.c. of saturated sodium carbonate solution. An intense blue color is gradually developed which will remain unaltered for

several days. Dilute the contents of both test-tubes to the 25 c.c. mark, and after at least 5 minutes make the color comparison in the usual manner.

The depth of the standard (in mm.) multiplied by 100 and divided by the reading of the unknown gives the sugar content, in mg., per 100 c.c. of blood.

Sample Analyses of Protein-Free Blood Filtrates Obtained by Means of Tungstic Acid.

No.	Mg. per 100 c.c. blood					
	Total N.	Urea N.	Uric acid.	Preformed creatinin.	Total creatinin.	Sugar.
1	26	10	1.3	1.5	6.0	89
2	26	13	1.0	1.4	5.3	100
3	28	12	1.1	1.2	6.7	98
4	28	12	2.2	2.0	5.7	83
5	29	13	3.3	1.5	6.0	86
6	29	11	2.6	1.4	5.2	95
7	29	13	1.6	1.4	6.0	85
8	30	13	2.4	1.6	5.5	82
9	30	14	4.1	1.7	5.3	82
10	32	15	2.8	1.6	5.4	91
11	32	15	3.4	1.4	5.3	97
12	32	13	2.4	1.7	6.0	104
13	33	17	2.0	1.3	4.8	83
14	33	16	2.5	1.6	5.7	105
15	33	15	1.1	1.6	5.5	95
16	34	16	0.8	1.3	6.1	119
17	34	16	2.6	1.5	5.9	106
18	35	17	2.1	1.6	6.0	89
19	35	17	2.0	1.4	5.5	77
20	35	18	2.0	1.7	5.7	86
21	35	18	2.9	1.6	5.8	95
22	35	17	3.2	1.4	5.5	94
23	35	18	2.5	1.5	6.0	89
24	35	19	2.2	1.5	5.3	91
25	35	22	3.5	1.4	5.7	87
26	35	17	2.3	1.7	6.7	83
27	35	18	1.6	1.3	6.5	104
28	36	17	2.8	1.5	5.2	100
29	37	18	2.1	1.5	5.5	94
30	38	18	2.2	1.7	5.4	95
31	39	18	2.6	1.8	6.7	103
32	39	18	2.9	1.5	6.0	87
33	40	18	2.0	1.6	6.0	98
34	40	20	2.6	1.7	5.6	95
35	41	19	4.8	1.5	5.9	93
36	41	19	4.2	2.5	6.6	109
37	43	19	2.2	1.7	6.3	78
38	139	106	5.4	12.5	19.4	99
39	147	115	8.9	11.0	20.5	170
40	275	237	14.3	13.6	27.2	157

The Determination of Ammonia in Blood.—*J. Biol. Chem.*, 11,-534.)—Reasonably accurate determinations of ammonia in blood are obtained with great difficulty because of the decomposition of certain nitrogenous components of blood even at room temperatures, and because the free ammonia actually present in fresh blood amounts only to a few hundredths of a milligram per 100 c.c.

Transfer 10 c.c. of blood to a large test tube. Add 2-3 c.c. of a solution containing 10 per cent. sodic carbonate and 15 per cent. potassium oxalate. Then aspirate the liberated ammonia by means of a rapid air current into a test tube containing 1 c.c. of water and 5-6 drops .1 N hydrochloric acid. Time 20-30 minutes. Nesslerize this solution by the gradual addition of not over 1 c.c. diluted Nessler's solution (dilution 1:5). Transfer the solution to a 10 c.c. volumetric flask, and dilute to volume with "ammonia free" water.

"Ammonia free" water is obtained from ordinary distilled water by the addition of a little bromin water and a few drops of concentrated sodium hydroxid.

The colorimetric valuation of the solution by means of the Duboscq colorimeter cannot be accomplished without materially altering the instrument. An iris diaphragm should be attached to one sliding platform of the colorimeter, so as to regulate the amount of light passing through on that side. The hexagonal prism should be removed from the opposite side. With these alterations, the Nesslerized solution in a 100 mm. polariscope tube may be compared with .5 or 1 mg. ammonia (Nesslerized and diluted to 100 c.c.). Place the standard in the cup on the side of the iris diaphragm, fill a 100 mm. polariscope tube with the unknown, and insert this on the other side. Adjust the standard until the two fields are equal.

Nessler's Reagent.—This reagent is essentially a solution of the double iodid of mercury and potassium ($HgI_2,2KI$) containing sodic or potassic hydrate. A stock solution of the double iodid is best prepared as follows:

Transfer 150 g. of potassium iodid and 110 g. of iodin to a 500 c.c. Florence flask; add 100 c.c. of water and an excess of metallic mercury, 140 g. to 150 g. Shake the flask continuously and vigorously for 7 to 15 minutes or until the dissolved iodin

has nearly all disappeared. The solution becomes quite hot. When the red iodin solution has begun to become visibly pale, though still red, cool in running water and continue the shaking until the reddish color of the iodin has been replaced by the greenish color of the double iodid. This whole operation usually does not take more than 15 minutes. Now separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a volume of two liters. If the cooling was begun in time the resulting reagent is clear enough for immediate dilution with 10 per cent. alkali and water, and the finished solution can at once be used for Nesslerizations.

The cost of the chemicals called for in this rather interesting process of making Nessler's solution is less than when starting with mercuric iodid and the disagreeable impurities present in many samples of mercuric iodid are avoided. From the stock solution of mercuric potassium iodid, made as described above, prepare the final Nessler solution as follows:

From completely saturated caustic soda solution containing about 55 g. of NaOH per 100 c.c. decant the clear supernatant liquid and dilute to a concentration of 10 per cent. (It is worth while to determine by titration that a 10 per cent. solution has been obtained with an error of not over 5 per cent.) Introduce into a large bottle 3,500 c.c. of 10 per cent. sodic hydrate solution, add 750 c.c. of the double iodid solution, and 750 c.c. of distilled water, giving 5 liters of Nessler's solution.

In the absence of modifying circumstances, such as the presence of much acid or alkali, this reagent should be added in the proportion of 10 c.c. per 100 c.c. of the volume to which the Nesslerized solution is to be diluted. As a general rule the volumetric flask (or volumetric test tube) should be at least two-thirds full before adding the Nessler reagent. If attention is not given to this detail turbid mixtures are obtained, and turbid solutions must never be used for color comparisons.

Preparation of Uric Acid and Phenol Reagent.—Transfer to a flask (capacity about 1500 c.c.):

750 c.c. of water,
100 g. of sodium tungstate,
20 g. of phosphomolybdic acid,
50 c.c. of phosphoric acid (85 per cent. H_3PO_4),
100 c.c. of concentrated hydrochloric acid.

Insert a funnel in the flask and partly close the opening of the funnel with a watch glass. Boil the mixture gently for two hours. A deep straw yellow solution should be obtained. It should not turn appreciably blue when a sample, 5 c.c., is rendered alkaline with sodic carbonate. Dilute to a liter.

Preparation of Uric Acid Reagent.—Introduce into a flask:

750 c.c. of water,
100 g. of sodium tungstate,
80 c.c. of phosphoric acid (85 per cent. H_3PO_4).

Partly close the mouth of the flask with a funnel and small watch glass and boil gently for two hours. Dilute to a liter.

Method for the Determination of Chlorids in Blood Plasma.—(*Rappleye: Jour. Biol. Chem.*, 1918, Vol. 32, p. 509). In this method the principle used in the Volhard Method for the determination of chlorids in urine is employed for the estimation of the minute amounts of sodium chlorid found in blood plasma.

The following solutions are required:

Solution I

Silver Nitrate	7.2653 gm.
Nitric Acid (concentrated)	250 c.c.
Saturated Solution of Iron-Ammonium-Alum......	50 c.c.
Distilled water to make.........................	1000 c.c.

Solution II

Potassium sulphocyanate in distilled water of such strength that 25 c.c. is exactly equivalent to 5 c.c. of the silver nitrate solution. Each c.c. of the silver nitrate solution is exactly equivalent to 2.5 mg. of sodium chlorid and each c.c. of the potassium sulphocyanate is equivalent to 0.5 mg. of sodium chlorid.

Procedure.

Place 2 c.c. of citrated plasma (oxalated plasma cannot be used on account of the poor end point) in a 50 c.c. volumetric flask containing 30 c.c. distilled water. Add 10 c.c. of Solution I and make to mark. After being mixed the liquid is allowed to stand for 5 to 10 minutes and is then filtered through a dry

filter paper free from chlorids. 25 c.c. of the filtrate is then titrated with Solution II.

To calculate the result subtract the number of c.c. of Solution II used in the titration from 25 and multiply by 50. This gives the number of milligrams of sodium chlorid present in 100 c.c. of blood plasma.

Nephelometric Method for the Determination of Fat in Blood (*Bloor*: *J. Biol. Chem.*, 17, 377).—Run 3 c.c. of blood slowly and with shaking into a 100 c.c. volumetric flask containing about 80 c.c. of a mixture of redistilled alcohol and ether (3:1). Raise the contents of the flask just to boiling (with constant shaking) in a water-bath, cool in running water, make up to the 100 c.c. mark with more alcohol-ether, mix, and filter into a small flask or bottle. Stopper tightly as soon as filtration is finished to avoid loss of liquid by evaporation.

Measure 15 c.c. of the filtrate, containing about 2 mg. of fat, into a small beaker, add 2 c.c. of N sodium ethylate, and evaporate the mixture just to dryness on the water-bath. To the dry residue add 5 c.c. of alcohol-ether (1:3), and warm gently until the flakes of alkali are loosened from the bottom of the beaker. To the mixture add 50 c.c. of water, and stir until a clear solution is obtained. Add 5 c.c. of a standard solution of oleic acid in alcohol-ether, containing about 2 mg. oleic acid, to 50 c.c. of water in another beaker. To the standard, and to the blood fat solution add (as nearly simultaneously as possible) 10 c.c. of 10 per cent. hydrochloric acid. Allow the suspensions so produced to stand for five minutes, and then compare by means of the Duboscq colorimeter previously converted into a nephelometer.

For the comparison, fill the two nephelometer tubes, after rinsing with the solutions, to the same height (the meniscus slightly above the dark collar at the top of the tubes), and place in the nephelometer, with the standard tube always on the same side. Set the movable jacket on the standard tube at a convenient point (30 mm. in the modified colorimeter described below), and make comparisons by adjusting the jacket on the test solution until the images show equal illumination. Make five readings alternately from above and from below, and take the average as the reading. Make the calculations in the same way as in the colorimetric methods, the values being inversely proportional to the readings.

Changing the Duboscq Colorimeter into a Nephelometer.—(*J. Biol. Chem.*, 22, 145).—A simple method for transforming the Duboscq colorimeter into a nephelometer is described in the *Journal of Biological Chemistry,* Vol. 22, p. 145, 1915. The extra parts necessary are supplied in an improved form by the International Equipment Company of Boston, Mass. By the use of these parts the change may be quickly made as follows: Unscrew the movable glass prisms of the colorimeter, slip the brass collars for the nephelometer tubes into place, and fasten on the plate from which the prisms were removed. Slip the movable jackets into the holes in the cup supports, and after pushing the nephelometer tubes into place in the collars, the instrument is ready for use. A darkened room and a light-tight box for the light are necessary. The box should be about 48 cm. long, 32 cm. high, and 20 cm. wide for the ordinary colorimeter. It should contain a bracket at one end to support the light (a 50 watt "Mazda") at the height of the nephelometer tubes, and a stop at the other end, against which the instrument may be pushed and so placed that the nephelometer tubes are about 30 cm. from the light. A slot in the top of the box to receive the telescope of the instrument and a dark curtain to cover the end of the box after the instrument is pushed into place complete the equipment of the box. All exposed parts should be painted a dull black.

Since the readings obtained from suspensions of different strength are not exactly proportional to the amount of precipitate present, it is necessary to calibrate the instrument for different strengths and make corrections accordingly. If, however, the solution to be tested is within 25 per cent. of the value of the standard, no correction is necessary.

A Method for the Determination of Cholesterin in Blood or Blood Serum.—The method consists in the application of the Autenrieth-Funk procedure (Autenrieth and Funk—*Münch, med. Wochenschr.*, 1913, Vol. 69, p. 1243) to the alcohol-ether extract of blood or serum prepared as for the determination of fat.

Measure 10 c.c. of the extract into a small beaker, and evaporate *just* to dryness on the water-bath or electric stove. (Any heating after dryness is reached produces a brownish color, which makes the determination difficult or impossible.)

Extract the cholesterin from the dry residue by boiling out 3 or 4 times with small portions (2-3 c.c.) of chloroform and de-

canting. Evaporate the combined extracts to a little less than 5 c.c., transfer to a 10 c.c. graduated cylinder, and make the volume up to 5 c.c. A little turbidity does not matter, since it disappears on adding the reagents. Measure 5 c.c. of a standard cholesterin solution in chloroform, containing .5 mg. of cholesterin, into a similar 10 c.c. graduate. Add to each 2 c.c. of acetic anhydrid and .1 c.c. of concentrated H_2SO_4. Mix the solutions by inverting two or three times, and set the cylinders in the dark for 15 minutes; then transfer the solutions to the colorimeter cups, and compare as usual, setting the standard at 15 mm.

The cement of the colorimeter cups must, of course, not be soluble in chloroform. Plaster-of-Paris has been found satisfactory, or even ordinary glue, if the cups are not used for any other purpose.

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